STUDIES OF SMALL INTESTINAL MUCOSAL FUNCTION AND THE INFLUENCE OF DISEASE IN MAN

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LET THERE BE LIGHT

Praise be to Allah, the cherisher and sustainer of the worlds, who has said in his Noble Book:

> *" Allah is the Light of the heavens and earth. The parable of His light is as if there were Niche and within it a Lamp, the lamp enclosed in Glass, the glass as it were a brilliant star. Lit from a blessed Tree, an olive, neither of the East nor of the West, whose oil is well-nigh luminous. Though fire scarce touched it: light upon light. Allah doth guide whom he will to his light. Allah doth set forth parables for people and Allah doth know all things."*

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PREFACE

One thousand year ago before the discovery of the circulation of the blood, and roughly thirteen centuries before it was known what happened in the intestine to ensure that organs were nourished by the process of digestive absorption, a verse in the Quran describes the source of the constituents of milk, in conformity with these notions.

To understand this verse, we have to know that chemical reactions occur in the intestine and that from there, substances extracted from food pass into the blood stream via a complex system, sometimes by way of the liver, depending on their chemical nature. The blood transports them to all the organs of the body, among which are the milk-producing mammary glands. Basically there is the arrival of certain substances from the contents of the intestine into the vessels of the intestinal wall itself, and transportation of these substances by the blood stream (Dr Maurice Bucaille) (1,2).

This concept must be fully appreciated if we are to understand the verse 66, chapter 16 in the Quran:

> " *Verily, in the cattle there is a lesson for you. We give you to drink of what inside their bodies, coming from a conjunction between the content of the intestine and the blood, a milk pure and pleasant for those who drink it.* "

SUMMARY

The idea behind this thesis developed during my employment at the Department of Gastroenterology, Glasgow Royal Infirmary University NHS Trust, as a Clinical Research Fellow. The department is one of the very few that has been established over the years as a centre for gastrointestinal research and investigations.

The studies I performed aimed to take advantage of the availability of techniques for the measurement of protein synthesis using stable (i.e non-radioactive) labelling methods and mass spectrometric analysis to provide comparison of the rates of incorporation of labelled amino acids into mucosal protein sampled by gastrointestinal biopsy when presented intravenously and intragastrically.

This project concerns itself with the potential role of the tracer amino acid infusion techniques in the investigation of gastrointestinal diseases particularly those associated with mucosal atrophy and hypertrophy.

The introductory section reviewed the anatomical and functional properties of the small intestine and the manifestations of small intestinal dysfunction (Chapter 1).

The second chapter discusses protein synthesis and metabolism in general particularly the nitrogenous body composition, components of protein metabolism and the control of various stages of protein synthesis.

The contribution of the small intestine to the whole-body protein metabolism is referred to in chapter 3.

Chapter 4 discusses the development of stable isotope technology with reference to it's advantages which led to it's wide use in the field of metabolic investigation. I have also mentioned the radionuclide isotopes and the disadvantages of their use in man. Various applications of stable isotopes were summarised in this chapter. At the end of this chapter I have outlined the reasons for undertaking this work explaining the hypothesis behind it and the areas that I wished to test.

In section II (Chapter 5) I have summarized my objectives and set out the aims of the various studies included in this thesis.

In chapter 6 I have discussed the principles of the methodology of stable isotope applications including gas chromatography mass spectrometry and isotope ratio mass spectrometry. This was followed by a brief explanation of the methods I used to obtain intestinal mucosal samples and the way I handled these biopsies for various measurements (Chapter 7).

Chapter 8 concerns itself with the development of a technique for the measurement of protein synthesis utilizing the tracer amino acid incorporation principle. The choice of amino acid tracer and of tracer delivery technique is discussed in this chapter.

The experimental design, recruitment of subjects and patients and laboratory work are discussed in chapter 9. I have explained the type of statistical analysis and the

methods I used to calculate the rates of protein synthesis and whole-body protein turnover at the end of this chapter.

Section IV concerns itself with the development of a new method of measurement of gastrointestinal mucosal protein synthesis. I have discussed the use of tracer amino acid delivered by primed-constant infusion to define the source of the precursor for protein synthesis and it's rate of incorporation when supplied by luminal (intragastric) and basolateral (intravenous) route in normal subject (Chapter 10). The time course of labelled amino acids incorporation into the mucosal protein was assessed as well (Chapter 11).

Section V outlines the application of this newly developed method in patients with untreated coeliac disease. I delivered two amino acid labelled with ^{13}C via the intravenous (IV) and intragastric (IG) routes and measured the rates of protein synthesis, whole-body protein turnover as well as the protein and nucleic acids composition of the gut mucosa (Chapter 12). I have also measured the crypt cell histomorphometric characteristics and compared them with the protein and nucleic acid composition of the mucosa. Correlation with vitamin B_{12} and folate status was assessed as well (Chapter 13).

The next application of the method described in this thesis was in patients with alcoholic liver disease as described in section VI (Chapter 14). I have measured the protein synthetic capacity and the protein/DNA ratio as well as the rates of intestinal mucosal protein synthesis and

whole-body protein turnover in this group and attempted to correlate these rates with the indices of disease severity.

The last application of this method in this thesis was in a group of ileostomy subjects (Section VII, Chapter 15). I have chosen this group of patients, with ileostomy after having total colectomy for the treatment of ulcerative colitis, to give me a practical, timely access to the terminal ileum. The principal aim for this study was to measure the rates of mucosal protein synthesis in the small intestinal mucosa both proximally and distally.

In section VIII I have given an overall discussion of the results obtained from various studies beside the discussion in each study in it's relevant section where the results are interpreted, commented upon, and appropriate deduction drawn.

Considerations on future applications of this method of measuring gastrointestinal mucosal protein synthesis as well as the uses of stable isotopes are commented upon in section IX.

General assessment of the results and conclusions are given in section X followed by references, publications arising from this thesis and presentations at various learned societies.

ACKNOWLEDGMENTS

I am particularly indebted to Dr R I Russell, Consultant in charge of the Department of Gastroenterology, Royal Infirmary, Glasgow for providing the facilities of the Gastroenterology Unit within the University Department of Medicine and in his encouraging and supportive role as a supervisor of the clinical and administrative aspects of this work.

My thanks are due to Professor M J Rennie, Symers Professor of Physiology, Department of Anatomy & Physiology, The university, Dundee who was of immense help. As well as advising on many aspects of this work, particularly the technical details and laboratory work, he was a great source of encouragement and reassurance.

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I would like to thank Dr A Shlebak, of the Haematology Department for his help in measuring vitamin B_{12} and folate.

I am very appreciative to the efforts of Laura Wasson, Margaret Straiton, Carole Campbell, Allison Cameron, Ali Taha and Walid Obiedat of the Gastroenterology Unit in providing technical and nursing assistance in performing the studies.

Acknowledgments are also due to several people who have been employed in the Department of Anatomy & Physiology, University of Dundee during the course of this work in particular Kenneth Smith and Shaun Downie for their laboratory technical assistance and advice.

I also would like to thank the Medical Illustration Department, Glasgow Royal Infirmary for the preparation of the microphotographs of the histological sections studied.

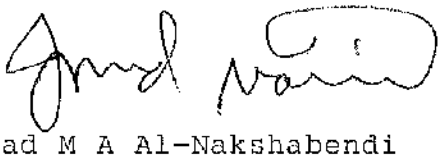
This work was supported by The Wellcome Trust, the University of Dundee, the UK Medical Research Council, The biomedical research Committee of the Chief Scientist of the Scottish Home & Health department and Wyeth Laboratories.

Finally, I would like to thank my wife, Eman, very much for her encouragement and patience during the course of this work.

D E C L A R A T I O N

I declare that the experiments described in the present thesis were carried out by myself in the Gastroenterology Unit, Glasgow Royal Infirmary University NHS Trust under the supervision of Dr Robin I Russell, except where acknowledged.

This thesis has been designed and composed by myself and is a record of work which has not been submitted previously for a higher degree any where.



Imad M A Al-Nakshabendi

I certify that the work reported in this thesis has been performed by Dr Imad M A Al-Nakshabendi, and that during the period of study he has fulfilled the conditions of the Ordinances and Regulations governing the Degree of Doctor of Philosophy.

Robin I Russell

ABBREVIATIONS

A	:	Area
a	:	Long radius
ALD	:	Alcoholic liver disease
ALT	:	Alanine transaminase
AMP	:	Adenosine mono phosphate
ANCA	:	Automated ^{15}N ^{13}C analyser.
APE	:	Atom Percent Excess
AST	:	Aspartate transaminase
ATP	:	Adenosine tri phosphate
BCAA	:	Branched-Chain Amino Acids
BDMS	:	Butyldimethylsilyl
BW	:	Body weight
b	:	Short radius
C	:	Carbon
^{0}C	:	Centigrade
cm	:	Centimeter
d	:	Day
DNA	:	Deoxyribo nucleic acid
e	:	Charge, electron
E	:	Enrichment
E_0	:	Baseline enrichment
E_p	:	Enrichment of the precursor
E_t	:	Enrichment of tissue protein at time t
E_{KIC}	:	Enrichment of the keto acid
eV	:	Charge in voltage
F	:	Rate of tracer infusion ($umol.kg^{-1}.h^{-1}$)
fl	:	Femtoliter

FSR	:	Fractional synthetic rate
GC	:	Gas Chromatography
GCMS	:	Gas chromatography mass spectrometry
GGT	:	Gamma glutamyl transferase
gm	:	Gram
GTP	:	Guanithine tri phosphate
h	:	Hour
H	:	Hydrogen
HCl	:	Hydrochloric acid
IG	:	Intragastrically
IRMS	:	Isotope Ratio Mass Spectrometry
IV	:	Intravenously
IU	:	International unit
K	:	Partition coefficient
k_d	:	Fractional degradation rate
kg	:	Kilogram
KIC	:	Ketoisocaproate
KIV	:	Ketoisovalerate
Kpa	:	Kilo pascal
k_s	:	Rate of protein synthesis, the rate parameter from the amino acid pool to the protein pool
l	:	Litre
LD_{50}	:	Lethal dose in 50% of exposed population
Leu	:	Leucine
M	:	Molar, molecular
MCV	:	Mean corpuscular volume
m/e	:	Mass-to-charge
m/z	:	Mass-to-charge ratio (ionized molecule)

mg	:	Milligram
min	:	Minute
ml	:	milliliter
mm	:	millimeter
mRNA	:	Messenger RNA
MS	:	Mass spectrometry
N	:	Nitrogen
ng	:	Nanogram
nm	:	Nanometer
O	:	Oxygen
P	:	Phosphorus
PDB	:	Pee Dee Belamnite; a carbonate from South Carolina used as an international standard for ^{13}C
pg	:	Picogram
Phe	:	Phenylalanine
PKU	:	Phenylketonurea
PCA	:	Perchloric acid
R, R'	:	Radical
RBC	:	Red blood corpuceles
RF	:	Ratio frequency
RNA	:	Ribonucleic acid
rRNA	:	Ribosomal RNA
RPM	:	Revolution per minute
SD	:	Standard deviation
SE	:	Standard error
s	:	Second
S	:	Sulphur

SVA	:	Subtotal Villous Atrophy
t	:	Time
t-BDMS	:	t-Butyldimethylsilyl
tRNA	:	Transferr RNA
ug	:	Microgram
umol	:	Micromole
UV	:	Ultraviolet
Val	:	Valine
Y	:	Year

SECTION I

INTRODUCTION

CHAPTER 1

THE SMALL INTESTINE AND IT'S FUNCTIONS

The gastrointestinal tract is a coordinated structure with the function of ingesting and absorbing nutrients and excreting unabsorbed and waste products. Knowledge of the small intestinal structure is important to understand the small intestinal mucosal functions, including protein synthesis and absorption, fully. The main anatomical and physiological properties of the small intestine are presented below.

1.1.1 STRUCTURE

The small intestine is a convoluted tube comprising the duodenum, jejunum and ileum extending from the pylorus to the ileocaecal valve where it joins the large intestine. During life it's average length is 5.92 m in women and 6.37 m in man and shortly after death becomes longer owing to the absence of muscle tone (3). On cross section it is consistent of mucosal layer, muscular layer and serosa (Figure 1.1.1). The mucosal layer composed of many cells, the enterocytes, which have a relatively short life span and rapid turnover, being formed in the crypts and then migrating to the villus (Figure 1.1.2) (4,5).

1.1.2 FUNCTIONS

The small intestine has two principal functions; digestion and absorption (5,6).

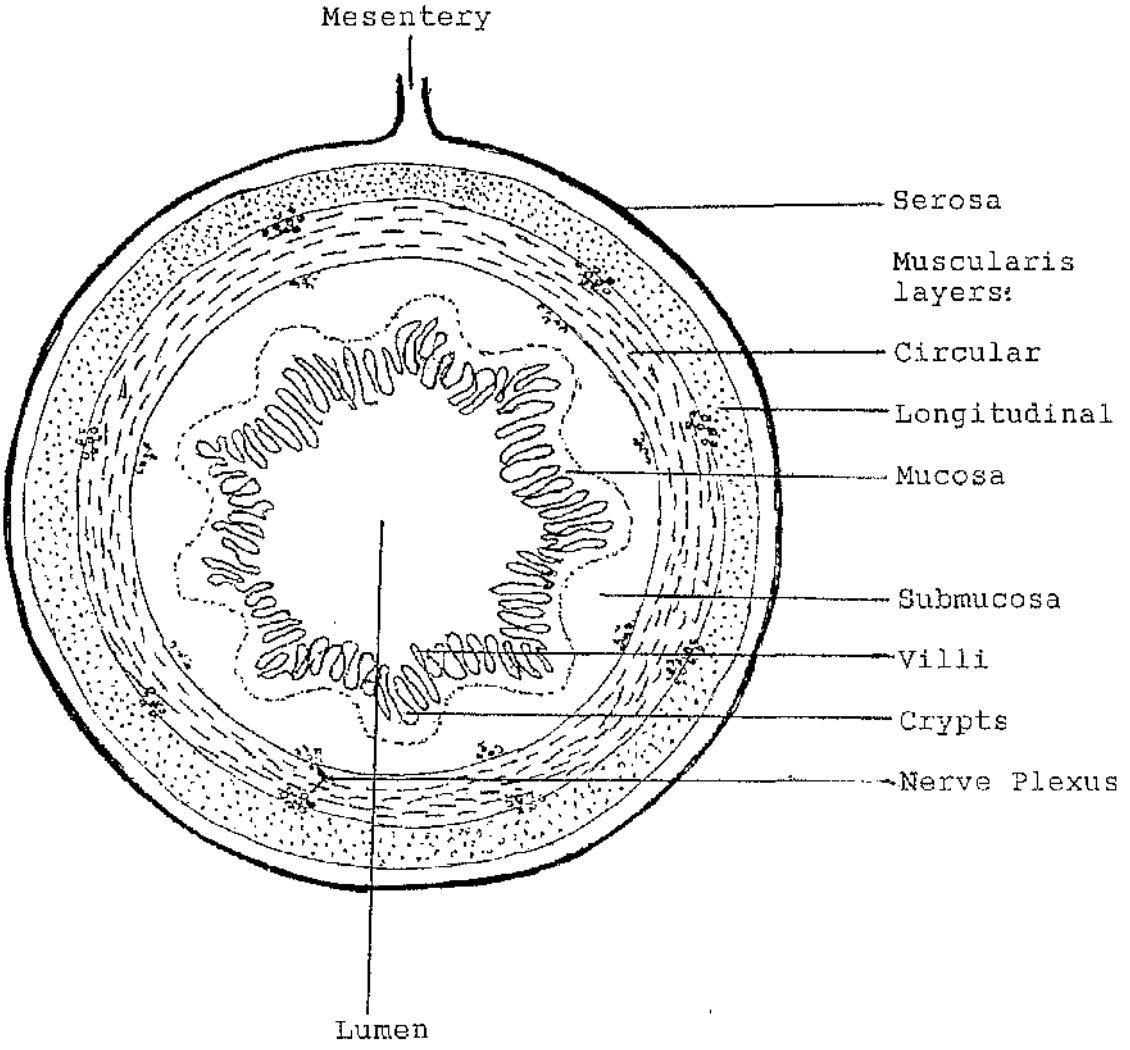


Figure 1.1.1 Schematic diagram of a cross section of the small intestine.

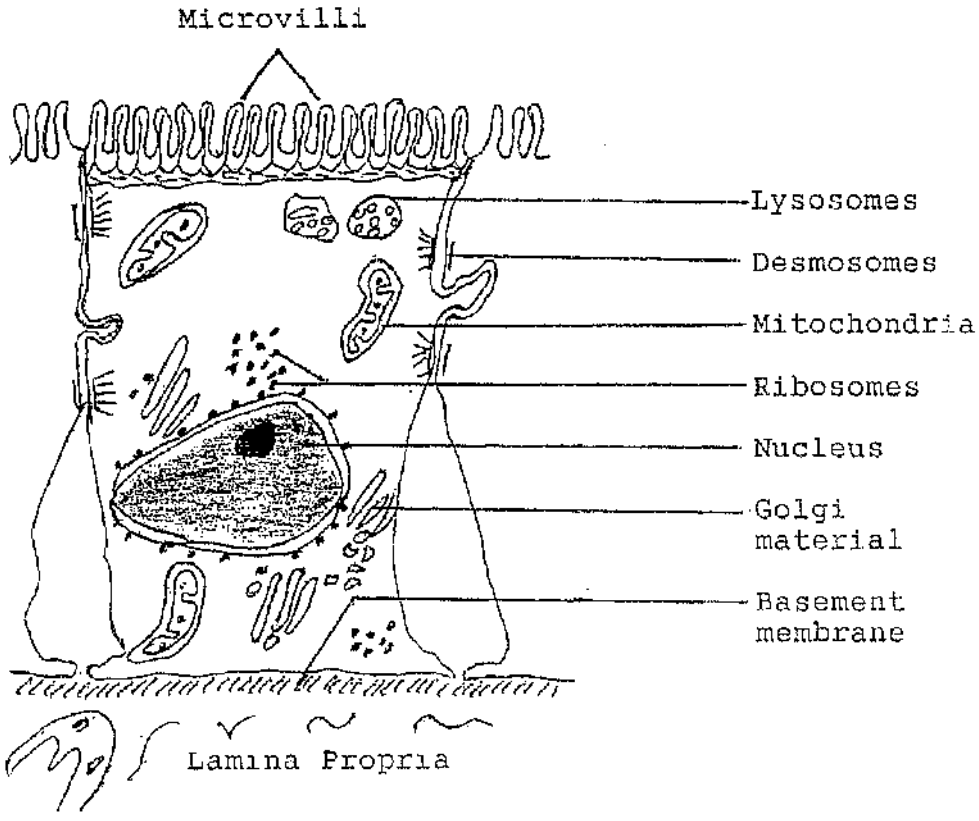


Figure 1.1.2 Schematic diagram of an enterocyte.

1.1.2.A DIGESTION:

The basic process of digestion is hydrolysis. Diet is mainly composed of carbohydrates, fats, proteins, vitamins, minerals and trace elements (7,8).

1.1.2.A.1 CARBOHYDRATES

The carbohydrates which are in the form of polysaccharides (starch) and disaccharides (sucrose, lactose & maltose) are digested into their monosaccharides (glucose, galactose & fructose). This process starts in the mouth where ptyalin is secreted in the saliva then hydrochloric acid (HCl) in the stomach provides some hydrolysis. However the major share of hydrolysis occurs in the small intestine under the influence of pancreatic amylase. Finally the disaccharidases, maltase, sucrase & lactase, of the brush border of the intestinal villus epithelium split the disaccharides into their corresponding monosaccharides which are absorbed into the portal blood.

1.1.2.A.2 FATS

Fats of the diet composed mainly of triglycerides which are neutral fats. Each molecule consist of glycerol and three fatty acids. There are small quantities of phospholipids, cholesterol and cholesterol esters. Very minute amount of fat can be digested in the stomach by the gastric lipase. Between 95 to 99 per cent of all fat digestion occurs in the small intestine under the influence of pancreatic lipase. The first step in fat digestion is emulsification, breaking the fat

globules into small size, which is achieved under the influence of bile salts that are secreted in the bile by the liver. The fat is split into monoglycerides and fatty acids. The intestinal epithelium contains a small quantity of lipase called enteric lipase which probably causes a very slight additional amount of fat digestion.

1.1.2.A.3 PROTEINS

Protein digestion begins in the stomach with the action of pepsin. The stomach contributes to only 10 to 20 per cent of total protein digestion. Most ingested protein is hydrolyzed in the duodenum and upper jejunum by the pancreatic enzymes trypsin, chymotrypsin and carboxy polypeptidases. The final product of this digestion is mainly small polypeptides plus a few amino acids. Oligopeptidases of the intestinal brush border hydrolyse di, tri and tetrapeptides. Although peptides consisting primarily of glycine, proline, hydroxyproline or dicarboxylic amino acids, they may be absorbed in peptide form, after which hydrolysis may occur within the mucosal cell. About 98 per cent of the protein finally becomes amino acids and the remaining 2 per cent is excreted in the faeces.

1.1.2.B ABSORPTION:

The small intestine plays the major role in absorption which occurs through the intestinal cells in various ways depending on the nutrient concerned. The stomach is a poor absorptive organ, only a few highly lipid soluble substances are absorbed in small quantities such as alcohol and some

drugs. The total amount of fluids entering the small bowel is about 8 - 9 l, most of it is absorbed leaving 1.5 l to pass through the ileocaecal valve. The colon absorbs most of this fluid leaving only 50 - 200 mls to be excreted in the stool.

1.1.2.B.1 THE ABSORPTIVE AREA

The small intestinal mucosa is shaped in the form of folds called valvulae conniventes which increase the surface area of the mucosa by about three-fold. Millions of villi projecting from the intestinal mucosa for about 1 mm increasing the absorptive surface area by another ten-fold. The mucosal epithelial cells are characterized by a brush border consisting of microvilli. There is about 600 microvilli from each cell. These microvilli increase the absorptive surface area by another 20-fold. Therefore the small intestinal mucosal absorptive area is increased by about 600-fold to a total of about 250 square meters (9).

1.1.2.B.2 MECHANISMS OF ABSORPTION

Absorption through the gastrointestinal mucosa occurs by active transport and by diffusion. Active transport provides energy to move a substance across a membrane against a concentration gradient or against an electrical potential. Diffusion means transport of substance through a membrane along an electrochemical gradient (10,11). The absorption of water, electrolytes and various nutrients is illustrated in (Figure 1.1.3).

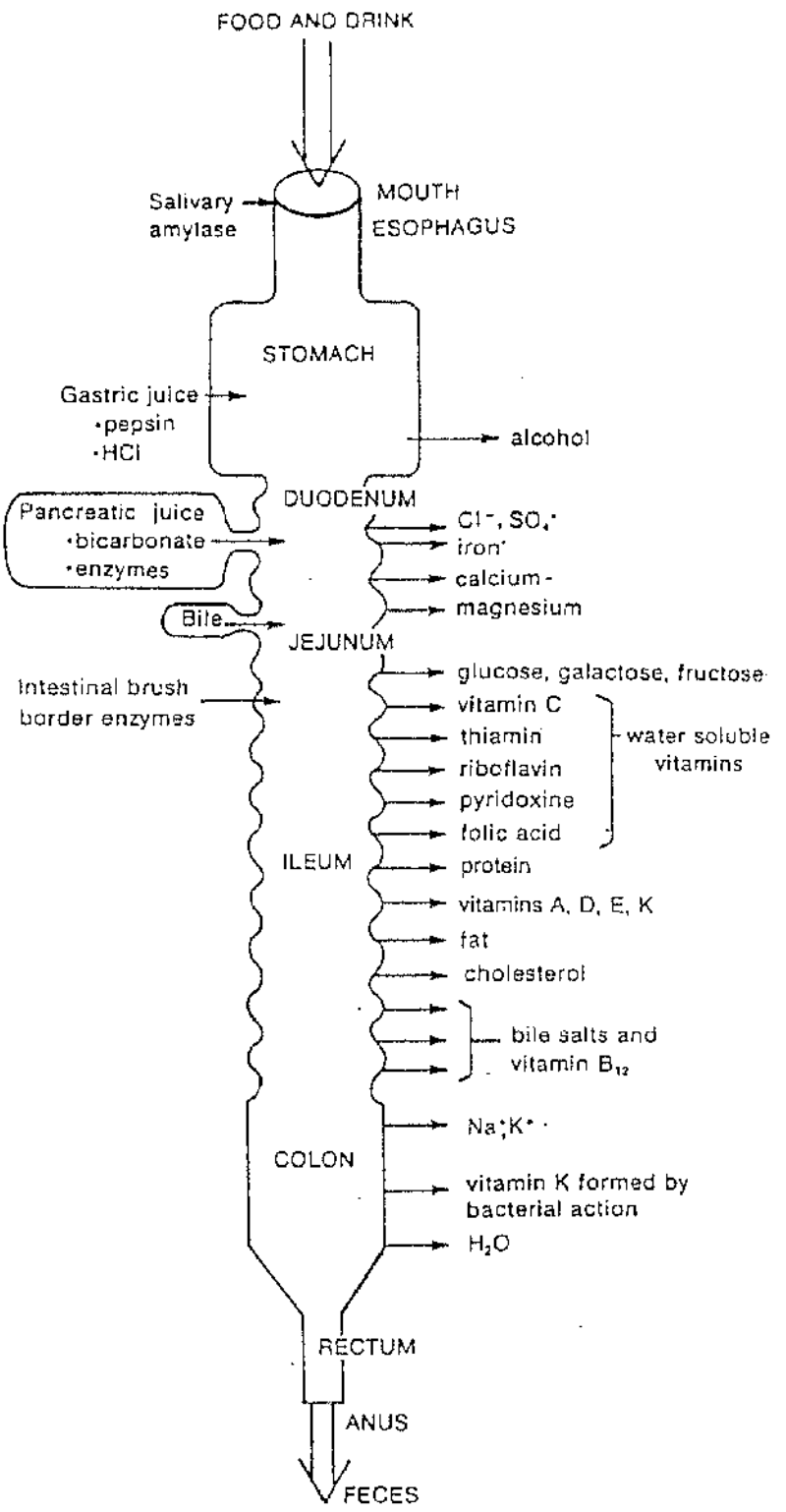


Figure 1.1.3 Gastrointestinal secretions and absorption.

1.1.2.B.3 WATER AND ELECTROLYTES

Water is transported across the intestinal membrane by diffusion which obeys the osmosis laws. As ions and nutrients are absorbed, an isosmotic equivalent of water is also absorbed. Electrolytes such as sodium, iron, potassium, magnesium, phosphate and other ions are actively absorbed. Chloride ions are transported in most parts of the small intestine along with the positively charged sodium ions. In the distal ileum and in the colon there is active transport of chloride ions as well.

1.1.2.B.4 CARBOHYDRATES

Carbohydrates are absorbed actively in the form of monosaccharides across the intestinal membrane together with the sodium ions. The energy required is provided by the sodium transport system; the so called sodium co-transport theory.

1.1.2.B.5 FATS

Fat digestion end products, free fatty acids and monoglycerides, become dissolved in the lipid portion of the bile acid micelles which are soluble in the chyme. Once these micelles are in contact with the surfaces of the intestinal epithelial cells, both fatty acids and monoglycerides immediately diffuse through the epithelial membrane leaving the bile acid micelles still in the chyme. After entering the epithelial cells these fatty acids and monoglycerides are taken up by the smooth endoplasmic reticulum and recombined

to form new tri-glycerides which aggregate into globules along with absorbed cholesterol, phospholipids and small amounts of synthesized cholesterol. These globules then are excreted to the spaces between the cells by exocytosis; from there they pass into the lymph and now called chylomicrons.

1.1.2.B.6 PROTEINS

Protein absorption is an active sodium co-transport system which is similar to the glucose transport mechanism. Dietary protein assimilation is preceded by acid denaturation in the stomach which renders the stable protein structure susceptible to hydrolysis, then luminal digestion producing small peptides and free amino acids (12). Protein absorption occurs mainly in the proximal jejunum (13). Most peptides and amino acids bind with a specific transport protein. There are many different types of these proteins in the intestinal epithelium. Amino acids enter the intestinal cell from the luminal side of the cell towards the basolateral side (6,10).

1.1.3 AMINO ACIDS TRANSPORT

Movement of amino acids into and out of cells is mediated by plasma membrane-bound activity which is an integral part of the small intestinal mucosal cells (14). Amino acids in the blood come from the products of enteral peptides digestion and luminal absorption as well as a result of protein and amino acid metabolism in organs throughout the body (15).

Free amino acids are transported across mucosal membranes via Na^+-independent (facilitated transport) and Na^+-dependent (secondary active transport) systems with only a small portion through unsaturable mechanism or diffusion (16,17,18,19). The definition of these systems is complicated by the presence of multiple transport systems within each group (20). Small intestinal amino acid transporters play a major role in whole body nitrogen metabolism during both absorptive and post absorptive states (14). In the absorptive state, enterocyte's apical and basolateral membranes transfer sodium and/or amino acids from the lumen to the portal blood in a way that sodium and water net absorption can be energetically coupled to amino acid uptake (21,22). Several of the apical (luminal) transport systems are different from those found on the basolateral membranes and are unique to absorptive epithelia. All the basolateral membrane systems are found else where in the body (15,23). In the post absorptive state circulating solutes are transported through the intestinal basolateral membranes into and out of enterocytes participating in splanchnic intermediary metabolism reaction (15). Some of the transport systems in the intestinal mucosa serve particular amino acids such as aspartate and glutamate (24), proline (25,26), neutral and cationic amino acids (26,27), neutral amino acids only (18,25). A recent study (28) shows that sodium dependent phenylalanine transport occurs only via a specific system called system B rather than a selective system previously designated PHE (29).

1.1.4 DYSFUNCTION

Small intestinal dysfunction may result in maldigestion; impaired nutrients hydrolysis and malabsorption; defective mucosal absorption of nutrients. In clinical practice malabsorption is used as a global term to include both aspects of dysfunction (30).

1.1.4.A MECHANISMS AND CAUSES

1.1.4.A.1 MALDIGESTION

Maldigestion is due to digestive enzymes deficiency (pancreatitis, pancreatic carcinoma, parenchymal liver disease, cholestatic jaundice), inactivation (Zollinger-Ellison syndrome, bacterial overgrowth, drug effects) or rapid transit as in post-gastric surgery.

1.1.4.A.2 MALABSORPTION

In malabsorption there is a damage to the actual absorptive process itself which may follow damages to the small intestinal mucosa such as in coeliac disease, tropical sprue, Crohn's disease, radiation damage, ischemia, infections, infiltration, AIDS and Whipple's disease.

1.1.4.B CLINICAL PICTURE

The degree of impairment of absorption has to be profound before clinically significant malabsorption will occur (12). The main symptom of malabsorption is diarrhoea which can be watery. The stool is bulky, pale and offensive. It tends to float and is difficult to flush owing to the increased gas

content rather than fat (31). Weight loss is a common manifestation of malabsorption and in children there is growth retardation and failure of sexual development. Widespread nutrients deficiencies may result in various symptoms and signs (Table 1.1.1) (30).

1.1.4.C INVESTIGATIONS

Investigations should be undertaken;

a. to confirm the diagnosis,
b. to asses the severity and the extent of malabsorption and
c. to identify the underlying cause.

The intestine is more difficult to investigate as compared to other organs such as liver, kidney and heart. There are no simple tests which reflect intestinal function and integrity in the same way as liver function tests, urea and electrolytes or cardiac enzymes. Therefore many indirect function tests have been developed.

1.1.5 EFFECT OF FEEDING ON ABSORPTION

In the absence of luminal feeding, as in the case of total parenteral nutrition (TPN) the absorptive capacity of the intestine is severely compromised (32), accompanied by gradual intestinal atrophy (33). On the other hand, enteral feeding, particularly of amino acids or proteins promotes the morphological, digestive and absorptive integrity of the mucosa (34), therefore this route for nutrition should be chosen whenever possible.

TABLE 1.1.1 Clinical manifestations of malabsorption.

MALABSORBED NUTRIENT	SYMPTOMS AND SIGNS
Fat	Fatty stool, watery diarrhoea, weight loss
Protein	Weight loss, muscle wasting, oedema, leuconychia.
Carbohydrate	Watery diarrhoea, flatus, abdominal distention, borborygmi.
Iron	Anaemia, glossitis, koilonycia, aphthus ulcers.
Folic acid	Anaemia, glossitis, aphthus ulcers.
Vitamin B_{12}	Anaemia, glossitis, neurologic disorders.
Vitamin A	Hyperkeratosis, night blindness.
Vitamin D	Rickets, osteomalacia, proximal myopathy.
Vitamin K	Bruising, bleeding.
Calcium	Parasthesia, tetany.
Magnesium	Parasthesia, tetany.
Zinc	Acrodermatitis, poor wound healing, poor taste.
Bile salts	Watery diarrhoea

CHAPTER 2

PROTEIN SYNTHESIS AND METABOLISM

Protein and amino acids metabolism encompasses protein turnover, amino acids oxidation and overlap with carbohydrate and fat metabolism (35,36).

1.2.1 PROTEIN TURNOVER

Turnover in general term means the process of renewal or replacement of a given substance. This process may either involve the production of newly formed material with the disappearance of some material already present or it may represent the exchange of material between two or more compartments (37). The protein turnover means the synthesis and breakdown of protein i.e protein exchange with a pool of amino acids (35,37). The term breakdown should be restricted to the degradation of protein into amino acids and the word catabolism is best avoided as it suggests an oxidative process. Thus the catabolism of amino acids to urea must be distinguished from the breakdown of protein to amino acids (37).

1.2.2 BODY COMPOSITION

Quantitative knowledge of the amount of protein and amino acids in the body or an organ at a given time is difficult to obtain. Most of the methods for measuring the protein concentration and composition of the tissue in living subjects are indirect and based on assumption (38). Methods

based on tissue biopsy are valuable and access to various tissues is nowadays possible. Among the tissues which have been sampled for such studies are gut and liver (39), breast (40), bone (41), skin and muscle (42). Although protein can be used as a fuel if they are not needed for protein synthesis (43), as in the case of carbohydrate and fat, it is mainly involved in the structure and function of cell (35). Surplus amino acids can not be stored nor are they excreted, rather used as fuel (43).

1.2.3 COMPONENTS OF PROTEIN METABOLISM

The dynamic nature of protein metabolism has been recognized for many years (44,37). Figure 1.2.1 shows the estimates of various components of protein metabolism. The rates of interchange of amino acids between various components were difficult to measure, therefore many workers depended on nitrogen balance to study these components of protein metabolism. They calculated the net difference between protein intake expressed as nitrogen content and nitrogen content of the body waste. These methods can give incorrect results if used less than obsessively and it's use in free-living human being is limited (45). With the development of stable isotope technology, accurate measurements of the rate of protein turnover and amino acid interchange between various compartments becoming possible and increasingly easier (46).

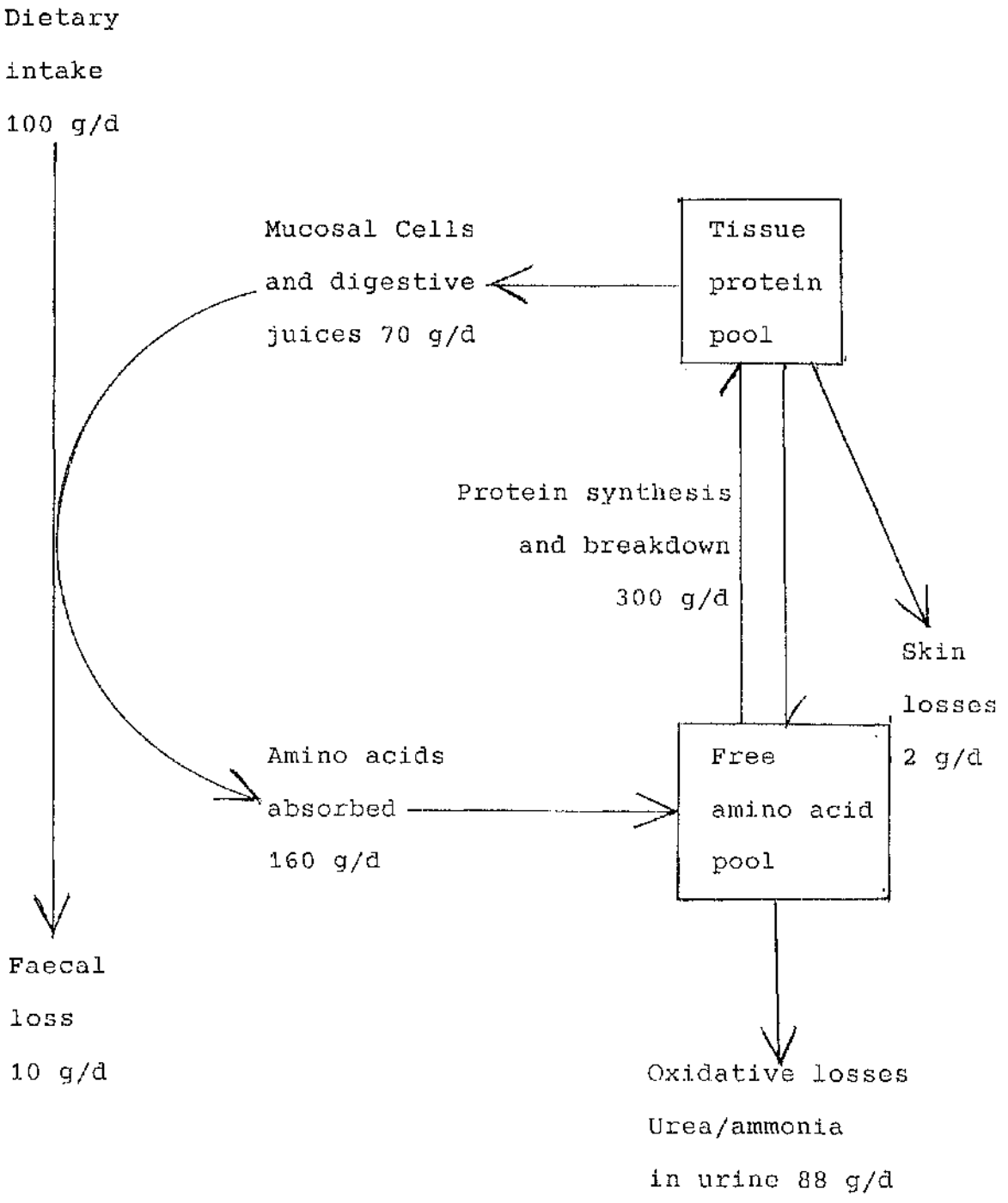


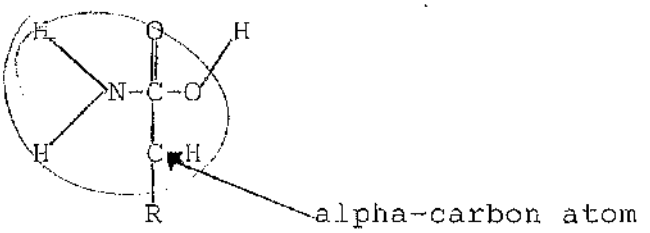
Figure 1.2.1 The components of protein metabolism.

1.2.4 THE AMINO ACIDS

Amino acids are organic compounds containing both an amino group and a carboxyl group as expressed in the following formula;

$$RCH(NH_2)COOH$$

alpha-amino acids are occurring in protein while in nature we have beta and gamma as well. The core of an alpha-amino acid is the carbon atom next to carboxylic group.



There are two optical isomers of amino acids in natural proteins L and D. All amino acids are asymmetric with the exception of glycine. There are four different groups attached to alpha-carbon atom; hydrogen atom, carboxyl group, amino group and R group and these groups are mirror images of one another and rotate polarized light in different directions (47).

1.2.4.A CLASSIFICATION

The principal constituents of proteins are amino acids, 20 of which are present in the body and are physiologically important (Table 1.2.1). Many of these amino acids are compounds absorbed by the intestine from dietary protein

TABLE 1.2.1 Concentrations of amino acids in plasma and RBC.

AMINO ACID	SYMBOL	PLASMA*	RED BLOOD CELLS**
ESSENTIAL			
Histidine	His	87 ± 3	120 ± 18
Isoleucine	Ile	63 ± 3	71 ± 3
Leucine	Leu	120 ± 5	137 ± 6
Lysine	Lys	195 ± 9	177 ± 5
Methionine	Met	25 ± 1	20 ± 3
Phenylalanine	Phe	53 ± 2	62 ± 2
Threonine	Thr	128 ± 5	157 ± 6
Arginine	Arg	86 ± 3	258 ± 23
Valine	Val	220 ± 8	248 ± 9
Tryptophan	Trp	43 ± 2	13 ± 1
NON-ESSENTIAL			
Alanine	Ala	316 ± 17	419 ± 16
Asparagine	Asn	47 ± 2	156 ± 4
Glutamate	Glu	-	446 ± 85
Glutamine	Gln	655 ± 17	758 ± 15
Glycine	Gly	248 ± 13	544 ± 21
Tyrosine	Tyr	60 ± 4	82 ± 5
Serine	Ser	114 ± 4	211 ± 6
Ornithine	Orn	66 ± 4	271 ± 18
Taurine	Tur	49 ± 3	157 ± 28
Citruline	Ctr	34 ± 1	48 ± 2

* umol/l ± SE, plasma water

** umol/l ± SE, intracellular water

Source; M Rennie (35)

digestion. Generally speaking these amino acids are divided into two main classes essential (dispensable) and non-essential (non-dispensable) amino acids (46,48). The essential amino acids could not be synthesized within the body in sufficient amounts to supply the demand of protein and nucleic acid synthesis (35). Strictly speaking the essential amino acids are isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, and histidine; in infants (35,48) and arginine; during growth (48). Arginine is synthesized by mammalian tissue but the rate is not sufficient to meet the needs during growth. In addition, if phenylalanine and methionine are present in only small amounts then tyrosine and cysteine may become rate-limiting to growth or function as methionine is required in large amount to produce cysteine and phenylalanine is required in large amount to produce tyrosine (35,48). The whole concept of essentiality has become much less rigid nowadays and the concept of "conditional essentiality" of certain amino acids was developed to take account of the fact that, despite the ability of the body to synthesize them, they are often required in amounts greater than the body's capacity to do so e.g glutamine, under conditions of disease (49,50) and injury and glycine in children treated for malnutrition (51).

1.2.4.B ANALYSIS

The introduction of automated amino acid analyzer made it possible to measure the concentration of various amino acids

in various tissues (Table 1.2.1). Individual amino acids may be analysed by a variety of enzymatic methods (52). There is a wide range of methods based on chromatographic separation (35), and more recently tracer methods both radioactive and stable isotope have been used successfully (46). Over 90% of the amino acids in the body are present in protein. The free amino acid pool contributes only a few percent to the total of amino acids in the body (53). The composition of the free amino acid pool in the plasma and within the intracellular compartment is the result of a variety of processes; amino acid synthesis, catabolism, transamination, protein synthesis, protein breakdown and amino acid transport. The complexity of amino acid metabolism prevents any attempt to partition the overall control of the free pool size between transport, intermediary metabolism and protein turnover (35). Nevertheless, factors which stimulate protein synthesis or inhibit protein breakdown seem to produce the largest changes in the free amino acid pool. Starvation is a good example (37), in which inhibition of protein synthesis and acceleration of breakdown markedly expands the free amino acid pool, particularly those amino acids with the smallest contribution to the free pool and thus the biggest protein bound/free ratio (e.g branched-chain amino acids, aromatic amino acids, and methionone). As can be predicted, feeding (which promotes anabolism) shrinks the pool whereas diabetes (which is catabolic) expands it.

1.2.5 GENETIC CONTROL OF PROTEIN SYNTHESIS

Cells and tissues are able to regulate their protein mass, the content of specific protein and the production of export protein in part by changes in the rate of protein synthesis **(37)**. Protein synthesis takes place in the cell cytoplasm and involves interactions between ribosomes, mRNA, tRNA, amino acids, ATP, GTP and various enzymes. Genes control reproduction and function of all cells including protein synthesis.

1.2.5.A STAGES OF PROTEIN SYNTHESIS

Protein synthesis and regulation are considered in two stages; transcription and translation.

1.2.5.A.1 TRANSCRIPTION

The DNA controls the formation of RNA which in turn controls the formation of a specific protein. DNA controls protein synthesis by means of " genetic code " which consists of successive triplets of bases, that is each group of three successive bases is a code word. The two strands of a DNA split apart, exposing the purine and pyrimidine bases projecting to the side of each strand. The purines are guanine and adenine and pyrimidines are thymine and cytosine. The successive triplets eventually control the sequence of amino acid in a protein molecule during it's synthesis in the cell. The genetic code is transferred from the DNA in the nucleus to the RNA. This process is called "transcription". The RNA then diffuses from the nucleus through the nucleolar

pores into the cytoplasmic compartment where it controls protein synthesis (10).

1.2.5.A.2 RIBONUCLEIC AIDS

Almost all DNA is located in the nucleus of the cell and yet most of the functions of the cell are carried out in the cytoplasm. This is achieved through the intermediary of another type of nucleic acid, RNA, the formation of which is controlled by the DNA of the nucleus. There are three types of RNA ;

a-Messenger RNA (mRNA)

These molecules, also called the "codons", carry the genetic code from DNA to the cytoplasm controlling the formation of the protein. They are made in the nucleus in the region known as the nucleoplasm and composed of several hundreds to several thousands of nucleotides in single strands. There are different codons for the 20 common amino acids found in protein molecules, also, one codon represents the signal for the start of manufacturing a protein molecule and three codons represent the signal to stop manufacturing a protein molecule.

b-Transfer RNA (tRNA)

All tRNA have similar basic clover-leaf structure with two binding sites, containing only about 80 nucleotides and are relatively small molecules in comparison with mRNA. Their molecular weight is about 25.10^3 daltons and they transfer

the activated amino acid molecules to the ribosomes to be used in assembling the protein molecules. Each type of tRNA combines specifically with one of the 20 amino acids that are to be incorporated into proteins. The specific code in the tRNA that allows it to recognize a specific codon is again a triplet of nucleotide bases and is called anticodon.

c-Ribosomal RNA (rRNA)

These rRNA constitute 60% of the ribosomes and form the matrix upon which protein molecules are actually assembled. The remainder of the ribosome is protein, containing about 75 different proteins that are both structural proteins and enzymes needed in the synthesis of protein molecule. Their molecular weight is up to 24.10^5 daltons and they are made in the nucleolus. The process of protein formation on the ribosomes is called "translation" (10,37).

1.2.5.A.3 TRANSLATION

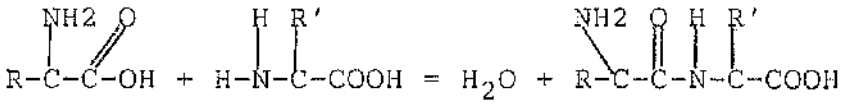
This process is divided into three phases;

(a) Initiation, binding of the ribosome to the mRNA and preparation for the synthesis of the first peptide bond in the peptide chain.

(b) Elongation, movement of the ribosomes along the mRNA as it incorporates amino acids into the growing peptide chain.

(c) Termination, completion of synthesis with release of the newly synthesized peptide chain and of the ribosome from the mRNA.

The successive amino acids in the protein chain combine together by peptide linkage according to the following chemical reaction:



In this reaction, a hydroxyl radical is removed from the COOH portion of one amino acid, while a hydrogen of the NH2 portion of the other amino acid is removed. These combine to form water and the two reactive sites left on the two successive amino acids bound with each other resulting in a single molecule.

For each peptide linkage formed during protein synthesis a very large amount of energy is consumed requiring breakdown of four molecules of ATP as shown in the following reaction:

$$\text{amino acid} + \text{tRNA} + \text{ATP} \xrightarrow[\text{synthetase}]{\text{aminoacyl-tRNA}} \text{aminoacyl-tRNA} + \text{AMP} + PP_i$$

Thus protein synthesis is one of the most energy-consuming of all cell's functions (10,37).

CHAPTER 3

CONTRIBUTION OF THE SMALL INTESTINE TO THE OVERALL PROTEIN METABOLISM

1.3.1 INTRODUCTION

The contribution of various tissues to whole-body protein turnover remains unknown. Each tissue must contribute according to it's mass, the amounts and composition of it's proteins and their turnover rates (54). Although the liver has the largest mass of amino acid catabolizing enzymes and containing the enzymes of urea cycle, other tissues such as the intestine, muscles, adipose tissue and kidneys also participate substantially in the intermediary amino acid metabolism of the whole-body and therefore the overall nitrogen economy (35). Protein turnover in the intestinal mucosa has various components (55). In the gut mucosa cell function is different at different levels. The physiology of absorption is not the same in the jejunum and ileum, the rates of cell renewal are not the same, and it is likely that rates of protein synthesis and breakdown are not the same (37).

1.3.2 COMPONENTS OF INTESTINAL PROTEIN SYNTHESIS

Protein synthesis in the gut has three components which serve different functions;

1.3.2.A CELL RENEWAL

The first component of protein synthesis in the small intestine is the cell renewal. The life span of the

intestinal epithelial cells is short, so that cell renewal must make a large contribution to protein synthesis in the tissue. In man the crypt cells proliferate at the rate of 1-2% per h (56). The turnover times of intestinal epithelial cells in man ranging from 3 days in the ileum to 5 to 6 days in the duodenum (4). Thus the fractional renewal rate would be at least 20% per day. The weight of the intestinal tract is 5% of the body weight (57) and if half of this is epithelium then the total mass of cells renewed per day would be about 350 gm in an adult man. This very rough estimate was confirmed by measuring the DNA content of the exfoliated cells in the intestinal perfusate (58). By this method it was estimated that the total weight of epithelial cells lost from the human gut would be 287 gm per day. If the cells have a protein content of 20%, this would mean 50-60 gm protein synthesized per day in the production of new cells.

1.3.2.B EXPORT PROTEIN

The second component of protein synthesis in the intestinal tract is the production of secreted or export proteins. It has been estimated that in an adult man about 17 gm protein is secreted per day through saliva, gastric juice, bile and pancreatic juice (57). In the crypts of the intestinal epithelium, the greater part of protein synthesis is for the production of new cells (59) whereas in the villi specific enzymes proteins are synthesized (55).

1.3.2.C INTRACELLULAR TURNOVER

The third component of protein synthesis in the gut is intracellular turnover. However there does not seem to be any way of measuring it directly in man. The combination of protein cell turnover and export protein amount to about 70-80 gm protein per day which is about one quarter of total body turnover in man. It seems that the intestinal tract clearly makes an important contribution to overall protein synthesis, probably more important than that of liver (37).

1.3.3 SOURCE OF AMINO ACIDS

The amino acid taken up into the epithelial cells is derived from two sources; the blood stream and the lumen of the gut. From animal studies it seems that there is a preferential uptake from one or other route according to the anatomical location of the cells (15,60). After IV administration of a precursor amino acid protein from cells near the villus-crypt junction is most heavily labelled whereas after intraluminal administration of the precursor protein from cells near the villus tip is most heavily labelled. Even if the labelled amino acids were infused by both route it would not be possible to achieve the same specific activity of enrichment in blood and in gut contents. By giving a prime dose, to flood the system, it would be possible to overcome this problem as after the bolus injection all the amino acids pools reach more or less the same specific activity or enrichment. However if the infusion continued for more than 6 h there will be more chance of

labelled protein to be broken down before the end of the incorporation period.

1.3.4 EFFECT OF NUTRITION

It seems that food intake stimulates protein synthesis and decreases protein breakdown in the fed state. In the fasted state, protein synthesis falls and protein breakdown rises. The swing between "gain" in fed state and "loss" in the fasted state is greatest when protein content of the diet is highest (35). It has been found recently that total parenteral nutrition (TPN) appears to increase rate of protein synthesis in the human gastrointestinal tract (39).

The whole-body protein dynamics and it's response to nutrient supply is now better understood after the application of tracer amino acid infusion studies (35).

CHAPTER 4

THE DEVELOPMENT AND POSSIBLE APPLICATIONS OF STABLE ISOTOPES

1.4.1 ISOTOPE TRACERS

Isotopes are atoms, (iso = same, topos = place; occupying the same place in the periodic table) (61), of different numbers of neutrons but the same number of protons i.e are members of the same element (46). Isotopes are used to label certain chemical compounds of interest to form a tracer. Tracer is a compound which is chemically identical to the compound of interest (the tracee), but distinct in some characteristic that enables it's precise detection. Therefore, isotope tracer is a tracer in which one or more naturally occurring atoms in specific position(s) substituted with an isotope of that atom with a less common abundance (46).

The chemical properties of the atom is determined by the electronic configuration and not the number of neutrons. The specifically enriched atom is referred to by identification of the weight of the atom by a superscript prior to the letter e.g ^{12}C, ^{13}C and ^{14}C refer to carbon atoms of atomic mass of 12, 13 and 14 respectively (^{12}C is the most abundant mass, ^{13}C is the stable isotope and ^{14}C is the radioactive isotope). The atomic mass number is the number of protons plus neutrons in the atom and the mass differences of isotopes are due to different numbers of nuclear neutrons. In our studies we used ^{13}C labelling of amino acids leucine and valine. ^{13}C and ^{15}N are the most widely used isotopes in metabolic studies (Table 1.4.1).

TABLE 1.4.1 Carbon and nitrogen stable isotopes.

ELEMENT	STABLE ISOTOPE	% OF NATURAL ABUNDANCE
C	12	98.89
	13	1.11
N	14	99.63
	15	0.37

The position within a molecule of an enriched carbon is referred to by the appropriate carbon number preceding the abbreviation of the description of the isotope used in the tracer, e.g 1-^{13}C-leucine refers to a molecule of leucine in which position 1 is labelled with carbon enriched with the stable isotope mass 13 (46).

1.4.2 STABLE ISOTOPES

These are the isotopes in which the neutrons are stable with no evidence of spontaneous degradation i.e they do not transmit into another element. The use of these isotopes as a metabolic tracer predates the use of radioactive isotopes by nearly 20 years (62,63), however, the analysis was by cumbersome techniques. It was probably the difficulty of this analysis that hindered the more widespread use of the stable isotopes (35). With the advent of scintillation

counting and the availability of a wide variety of radioactive tracers, most kinetic studies in the 1950s and 1960s used radioactive tracers (46). Over the past 15-20 years the use of stable isotopes have become increasingly popular, especially since the development of mass spectrometers interfaced with gas chromatography (35,46). Greater availability of stable isotopes as well as the increased awareness of the health hazards of radioactivity, also stimulated the use of stable isotopes (46).

1.4.2.A BIOLOGICAL ADVANTAGES:

The most obvious advantage of stable isotopes is that they are not radioactive and present little or no risk to human subjects (46). Side effects *in vivo* are not expected from administration of "tracer" doses of carbon 13 since carbon 13, naturally contributes to 1.11% of the carbon pool (Table 1.4.1), has not been found to demonstrate more than trivial *in vitro* isotope effects on chemical reaction with carbon 13-labelled substrate (64). ^{13}C and ^{15}N can be shown to affect certain parameters of cell function at extremely high levels of enrichment that would never be attained in an *in vivo* study in humans (65). There is no evidence that stable isotopes tracers present an identifiable risk to human subjects at the highest levels of enrichment that might reasonably be achieved. The use of label tracer requires the assumption that the labelled molecule will not be discriminated from the unlabelled molecules and that the labelled molecule will trace the movement of the unlabelled

molecules. Furthermore, there is little evidence that enzymatic effects are of concern in *in vivo* studies when the tracer atom is not directly involved in a metabolic reaction (46).

1.4.2.B ANALYTICAL ADVANTAGES:

In addition to the safety of stable isotopes for human use in metabolic investigations, there are other advantages of stable isotopes (66). Most obviously, the availability of stable isotopes for nitrogen and oxygen (^{15}N and ^{18}O) whereas there are no practical radioactive isotopes for these two elements. When analysis by gas chromatography mass spectrometry is used, it is often possible to determine enrichment on small samples of blood. There are possibilities of; adding an internal standard and thereby measuring the concentration and enrichment spontaneously; selective ion monitoring generally enables reliable documentation of the purity of a sample being analysed; simultaneous and repeated use of several tracers is possible in the same subject, since the process of mass spectrometry isolates individual compounds before analysis. Thus several amino acids enriched with ^{13}C can be given simultaneously and the enrichment of each tracer measured independently of the other labelled compounds; and it is frequently possible to determine the enrichment in specific position of a molecule.

In some of our studies we used two tracers simultaneously i.e ^{13}C-leucine and ^{13}C-valine in the same subjects to

determine the effect of route of delivery on the protein synthesis in normal subjects and in coeliac patients.

1.4.3 RADIOACTIVE ISOTOPES

Radioactivity means the spontaneous rearrangement of protons and/or neutrons in an unstable atomic nucleus in such a way that expandable energy becomes available. A proton may change into a neutron or vice versa, or an orbital electron may be captured by the nucleus, thus altering the nuclear charge (transmutation); alpha-particles, electrons, or positrons may be shot out at high speed; or the excess energy may be carried away by gamma-photons or conversion electrons, leaving proton and neutron numbers unchanged (isometric transition). There are characteristic decay processes and omitted radiations for each radionuclide. Radioactive isotopes are the isotopes in which neutrons spontaneously degrade to form an electron and a proton. Radioactive tracers of amino acids have been used successfully in tracing plasma amino acid turnover (35). Radioactive is an incorrect term and is better called radionuclide (61) which is a nuclide that spontaneously exhibits radioactive decay. Determination of ß radioactivity in a sample is done by scintillation counting. The technique allows simultaneous counting of ^{3}H and ^{14}C. Account must be taken of quenching of counts to convert from count per minute (observed by the instrument) to disintegrations per minute (46).

Most kinetic studies in the 1950s and 1960s used radioactive tracers prior to the advent of sensitive and

user-friendly mass spectrometers in various configurations (35).

1.4.3.A BIOLOGICAL DISADVANTAGES:

Administration of radioactive tracers involves health risk to the subjects. Therefore the dose of radioactivity must be calculated and compared with commonly encountered radiation exposures to gauge risk. Table 1.4.2 shows the significant risk to different systems after exposure to various doses of irradiation (67).

In many European countries, the use of radioactive tracers in normal, healthy subjects is severely limited by law; and even in those countries where the law restrictions are less severe, ethics committees and regulatory authorities rightly demand a very good case to be made for the use of radioactive tracer in adults of reproductive age (68), and it is impossible to study children (37).

1.4.3.B ANALYTICAL DISADVANTAGES:

The accuracy and precision with which metabolism can be traced using radioactive amino acids is usually less than with stable isotopes amino acids, because measuring the extent of labelling with radioactive amino acids involves two separate processes, i.e measurement of the rate of disintegration of radioactive isotope and the chemical concentrations of the tracer plus tracee. With stable isotope measurement methods, the quantity analogous to the specific

radioactivity is the isotope ratio and this can be obtained in a single measurement by a mass spectrometer. Liquid scintillation counters can be relatively inefficient way of measuring radioactivity; and this means that to obtain reliable measurements of incorporation of a label into a protein, a high dose of labelled amino acid would need to be used (69,70), unless prolonged counting times for the samples are used as a means of ensuring high precision.

1.4.4 FUTURE PERSPECTIVE

In the last 15-20 years, the developmental problems previously associated with employing stable isotope tracers for the metabolic investigations *in vivo* have largely disappeared. Furthermore, the use of stable isotopically labelled material became widely available and less expensive. The practical applications of these isotopes include the study of body composition, energy balance, inter-organ transport and oxidation of the three major metabolic fuels i.e glucose, fat and amino acids, the pathophysiology of metabolic events in growing children (66) and in disease conditions and in all branches of medical research. Table 1.4.3 shows the range of stable isotope applications in clinical research without attempting to be comprehensive.

TABLE 1.4.2 Risks related to radiation dosages.

EXPOSURE (RADS)	SYSTEM	SIGNIFICANCE
1	Bone marrow	Risk of leukemia 1/50 000
10	Sensitive organ	Risk of cancer 1/50 000
10	Whole-body	Increased number of chromosome aberration in peripheral blood, no detectable sign or symptom.
20	Reproductive system	doubling spontaneous mutation.
100 single dose	Whole-body	Mild irradiation sickness
650 single dose	Whole-body	LD_{50}

LD_{50} : lethal dose in 50% of exposed population.

TABLE 1.4.3 Clinical and research applications of stable isotopes.

APPLICATION	TRACER
Energy expenditure (71)	$^{2}H_{2}{}^{18}O$, $^{2}H_{2}O$
Total body water (72)	$H_{2}{}^{18}O$, $^{2}H_{2}O$
Protein turnover	
Whole body (73)	[^{13}C]leucine, [^{15}N] glycine, [^{15}N]lysine
(74)	($^{2}H_{5}$)phenylalanine
Tissue & individual proteins (75, 76)	[^{13}C]leucine, [^{15}N] glycine
Fat oxidation and turnover (77,78)	[^{13}C]palmitate, [$^{2}H_{5}$] glycerol
Carbohydrate oxidation, turnover(79)	[^{13}C]glucose
Amino acid kinetics	
Transamination (75,80,81)	[^{15}N,^{13}C]leucine
Pool size, turnover (82)	[^{15}N]glycine, [^{15}N] alanine, [^{13}C]leucine
Substrate cycling (83)	[$^{2}H_{5}$-fatty acids and glucose
Trace elements and mineral metabolism (84,85,86)	^{43}Ca, ^{44}Ca, ^{54}Fe, ^{57}Fe ^{204}Pb, ^{65}Cu, ^{70}Zn, ^{28}Mg
Inborn errors of metabolism	
Metabolic pathways elucidation(87)	Various
Heterozygot detection (PKU) (88)	[$^{2}H_{5}$]phenylalanine
Pharmacokinetics (89)	Various

TABLE 1.4.3 Clinical and research applications of stable isotopes.(continuation)

APPLICATION	TRACER
Breath tests	
Lactase deficiency (90,91)	[^{13}C]lactose
Fat malabsorption (92,93,94)	[^{13}C]-tri-glycerides and fatty acids
Liver function (95,96,97)	[^{13}C]aminopyridin, [^{13}C]galactose
Bacterial overgrowth (98)	[^{13}C]glycholate
Helicobacter pylori (99)	[^{13}C]urea
Gastric emptying (100,101,102)	[^{13}C]glycine / [^{14}C] octanoic acid
Cancer, liver (103)	[^{13}C]galactose
urinary bladder (104)	[^{13}C]tryptophan
therapy response (105)	deutrium labelled cytotoxic drugs.
Ileal dysfunction (106)	[^{13}C]-labelled glycocholate

It seems likely that within the near future the stable isotopes will totally replace radioactive isotopes in quantitative metabolic studies involving human subjects (107).

1.4.5 GASTROINTESTINAL MUCOSAL PROTEIN SYNTHESIS

Animal work suggested that the gastrointestinal tract contributes significantly to the whole-body protein metabolism. There has been a substantial interest in the gastrointestinal protein metabolism in man.

So far there have been no reliable, safe, robust methods for the measurement of the incorporation of tracer labelled amino acids into protein in the gastrointestinal tract widely available although there have been reports of measurement of tracer incorporation into normal and diseased gastrointestinal tract tissues on an *ad hoc* basis.

The purpose of the present work was to develop a method which would allow measurement of protein synthesis in any gastrointestinal mucosal tissue capable of being sampled.

In designing such a method, two questions of some significance need to be addressed. The first question is whether a preferential route of entry of amino acids to the synthetic precursor machinery exists in small intestinal mucosal tissue. This is an important question the answer to which has implications for the preservation of the function of the gastrointestinal tract during enteral and parenteral feeding. The second question is whether the tracer amino acid is incorporated into gastrointestinal protein to an extent proportional to the time of exposure of the tissue to the tracer i.e linear incorporation. The answer to this question would validate the method of calculating the rate of protein synthesis as a fractional rate.

Furthermore, since comparison will be made of the rates of protein synthesis in subjects without gastrointestinal disease and in others with, for example coeliac disease and alcoholic liver disease where the nutritional status is affected by the disease state, it will be possible to discover something about the likelihood that differential rates of protein synthesis are involved in pathological changes known to occur in the gastrointestinal mucosa in these conditions.

Since the investigation aims to discover new facts about normal physiology as well as it's derangement in pathological circumstances I would require to study patients with normal gastrointestinal tract function as well as others with deranged function. Thus I have chosen normal subjects and patients with untreated coeliac disease, alcoholic liver disease and subjects with ileostomy following total colectomy for the treatment of ulcerative colitis to give me access to the distal part of the small intestine.

These investigations aimed to take advantage of the availability of techniques for the measurement of protein synthesis using stable isotope labelling methods and mass spectrometric analysis.

SECTION II

AIMS OF THE STUDIES

CHAPTER 5

AIMS

The overall aim of the studies described in this thesis was to assess certain aspects of the small intestinal mucosal function in normal subjects and in certain disease conditions.

2.5.1 NORMAL SUBJECTS

The aims were;

1) to measure the gut mucosal protein synthesis,
2) to assess the protein, DNA and RNA composition of the intestinal mucosa,
3) to examine the effect of the route of amino acid delivery on the protein synthesis in the intestinal mucosa, and
4) to assess the pattern of amino acid incorporation into the mucosal protein in the small intestine.

2.5.2 COELIAC DISEASE PATIENTS

The aims were;

1) to assess the effect of untreated coeliac disease on the small intestinal mucosal protein synthetic rate and capacity and on the whole-body protein turnover,

2) to examine the effect of the route of amino acid delivery on the protein synthesis in the intestinal mucosa,
3) to compare the protein, DNA and RNA composition of the intestinal mucosa with the histomorphometric characteristics of the crypt cells in the intestinal mucosa, and
4) to investigate if vitamin B_{12} and folate status has any association with the crypt cell morphometry.

2.5.3 ALCOHOLIC LIVER DISEASE PATIENTS

The aims were;

1) to assess the effect of alcoholic liver disease on the small intestinal mucosal protein synthetic rate and capacity, and on the whole-body protein turnover,
2) to correlate the severity of alcoholic liver disease with the changes in the rates of small intestinal mucosal protein synthesis and whole-body protein turnover, and
3) to assess if the amount of consumed alcohol has an effect on the small intestinal mucosal protein synthesis and the whole-body protein turnover.

2.5.4 ILEOSTOMATES

The aims were;

1) to measure the rate of small intestinal mucosal protein synthesis in the proximal and the distal parts of the small intestinal mucosa in subjects with ileostomy,
2) to investigate if there is any variation in the rate of small intestinal mucosal protein synthesis between the various regions of the small intestine, and
3) to assess the effect of ileostomy on the whole-body protein turnover.

SECTION III

METHODOLOGY AND EXPERIMENTAL DESIGN

CHAPTER 6

STABLE ISOTOPES TECHNOLOGY

3.6.1 INTRODUCTION

The demonstration of the existence of isotopes of the element neon using mass spectrometry (MS) was first made by Sir J J Thompson in 1913 (107). Today the basic principle used to analyze stable isotopes remains unchanged.

Ashton laid down the ground work of stable isotope tracer studies by using the measurement of isotope ratio by mass spectrometry to establish the basis of the whole number rule of atomic weight in the 1930s (46). However, it was Schoenheimer et al. (108) in the early 1930s who was able to demonstrate the dynamic nature of protein metabolism in the body using isotope ratio mass spectrometry (IRMS).

The chromatographic system using gas to carry molecules through a sorbent filled column was suggested by Martin and Synge in their Nobel Prize winning study published in 1941 (109). The introduction of gas-liquid chromatography in 1950s (110) and it's subsequent interface with MS was a major breakthrough in analytical technology. The gas chromatography and mass spectrometry have proven to be well matched for combined operation, and advances in gas chromatography-mass spectrometry (GCMS) methodology have been the main reason behind the wider application of stable isotopes in metabolic research (35,46).

A schematic representation of the components of GCMS is shown in figure 3.6.1.

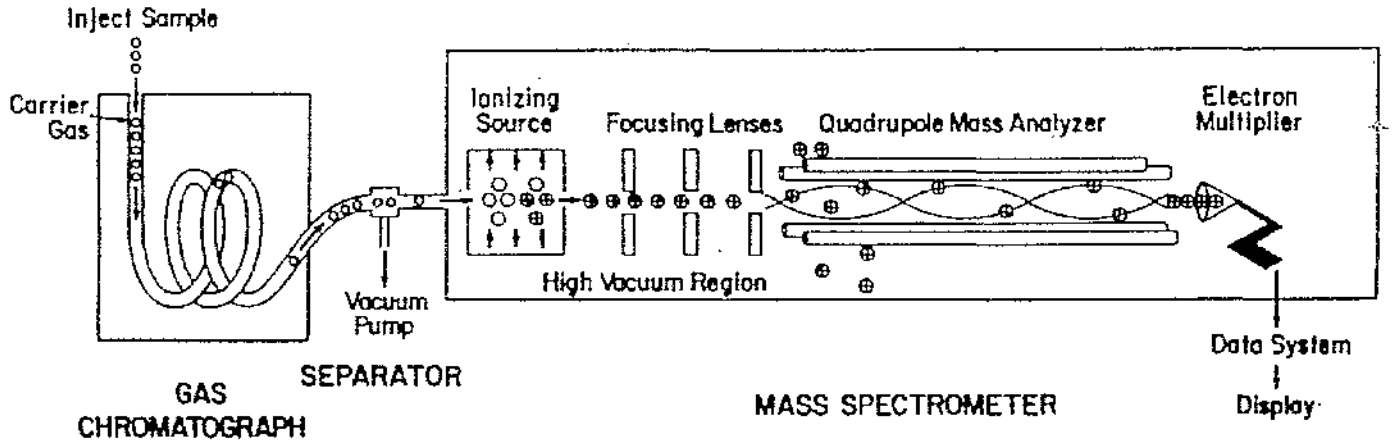


Figure 3.6.1 Schematic diagram of the components of a gas chromatography mass spectrometry system.

3.6.2 GAS CHROMATOGRAPHY

Chromatography is a method that separates mixtures of substances by their differential migration through a two-phase system consisting of a mobile phase (flowing gas or liquid) and a stationary phase (fixed porous sorbent). The primary function of the mobile phase is to promote the progression of analysts through the system and the primary function of the stationary phase is to imped selectively the progress of different analysts. The mobile phase in GC is a flowing inert carrier gas and in liquid chromatography is a chemically active solvent.

Separation of volatile compounds by GC is simple, rapid and reproducible method of high resolution. The sample is vapourised in the heated injector and swept through the column by an inert carrier gas such as helium, hydrogen, nitrogen or methane. The sample in the carrier gas is referred to as the gas phase or mobile phase. the separation process on the column occur in a temperature-controlled oven and effluent enters the MS interface.

3.6.2.A DERIVATIZATION

Most metabolic substrates in the body are not volatile, therefore to be separated by GC these compounds must be converted to derivatives that are thermally stable, chemically inert and volatile at temperature below about 300^0C. The strong intermolecular attractions between polar groups render most metabolic substrates non-volatile. Derivatization can markedly increase volatility by masking of

polar groups. Replacement of hydrogen in these groups by alkylation, acylation or silylation increases the volatility. There are important factors to be considered in choosing a derivative to use for GCMS analysis such as ease of preparation, stability of the derivatized sample, the separation characteristic on the GC, the resulting fragmentation pattern as well as prior use and documentation of particular derivative. Account must be taken of the extra atoms added by the process of derivatization and their contribution to the observed mass spectrometry data.

3.6.2.B PARTITION COEFFICIENT (K)

The injection port of the gas chromatogram is about 250^0C so that all compounds of the sample are vapourized. After injection the sample is partitioned between the inert carrier gas and a stationary phase which is a nonvolatile liquid coated onto the sides of the column or onto an inert, size-graded solid and packed into column. The different compounds in a sample will be present partially in the stationary phase and partially in the mobile phase depending on their vapour pressure. The ratio of the weight of solute per ml stationary phase (nonvolatile) to the weight of solute per ml mobile (gas) phase is the partition coefficient (K). A compound with a low K (high vapour pressure) will be present to a greater extent in the gas phase and come of the column (elute) more rapidly than will compounds with lower vapour pressure. The column temperature can be regulated to enable the elution of the compounds of interest in as short a period

as possible. The GCMS interface can also be heated so that the eluted compounds do not condense.

3.6.3 GC-MS INTERFACE

The carrier gas flow rate for a a GC is too high and at too great a pressure to enter into the ion source of the MS. The sample is also diluted with the carrier gas. Therefore a separator at the interface between GC and the MS is required to reduce the pressure and increase the relative amount of sample that enters the MS. The separator depends on the difference between the physical properties of the carrier gas and the sample.

3.6.3.A TYPES

Mainly there are three types of separators commonly in use i.e jet, effusion and indirect membrane separators (Figure 3.6.2).

(a) *Jet Separator;* The effluent from the GC passes through a small orifice into an evacuated tube. Increased concentration of the sample gas is achieved by pumping the lighter carrier gas away from the heavier component i.e the sample in the jet stream. The sample continues into a collecting capillary that leads into the ionization chamber of the MS.

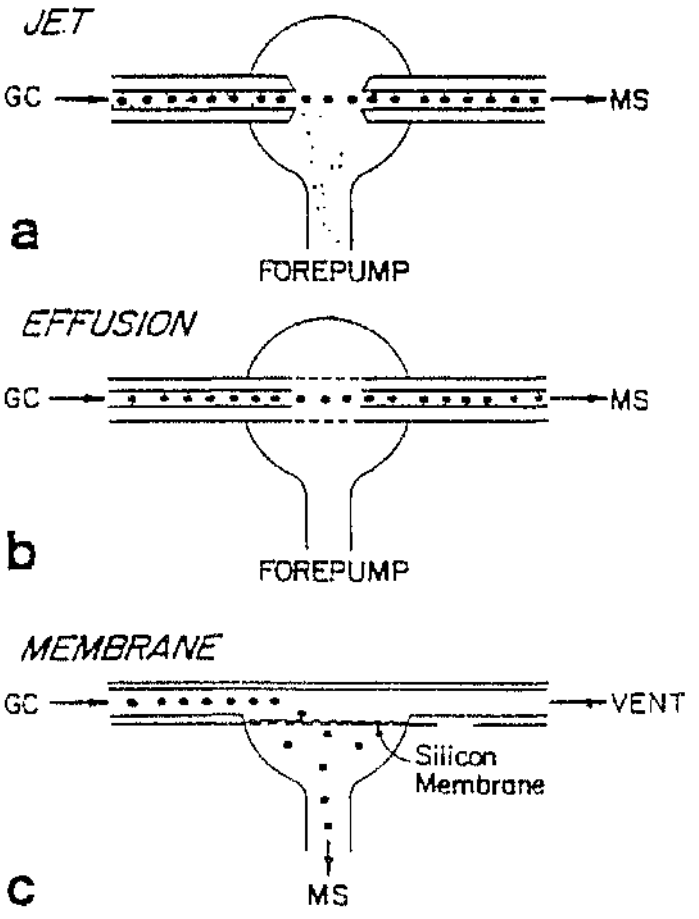


Figure 3.6.2 Schematic drawing of three separators used to interface GC with MS.

(b) *Effusion Separator;* The effluent from the GC passes through an inlet constrictor that reduces pressure from approximately 760 to 1 Torr. The carrier gas is separated from the sample because the rate at which the gas effuse through the glass frit to the external vacuum is inversely proportional to the square root of the molecular weight and directly proportional to the partial pressure of each component. The concentrated stream then passes to the ionizing chamber through a second restrictor and the carrier gas will preferentially effuse through narrow passage in a porous glass frit and be pumped away by a forepump.

(c) *Indirect Membrane Separator;* The preferential passage of organic vapour through a silicone polymer membrane concentrates a sample when a membrane separator is used. The carrier gas is insoluble in the polymer and is therefore pumped out, while the vapour of sample can pass directly into the MS. This type of separator is temperature dependent and there is also a significant time lag between the entry of compounds and their transfer to the MS which made the membrane separator less favoured than the other types of separators.

Some MS are designed so that the GC effluent enter in the ion source directly so that prior sample separation is not required. This is particularly common when a capillary GC is used, since the carrier gas flow rate is so much less than when a packed-column GC is used.

3.6.4 MASS SPECTROMETRY

The basic principle of mass spectrometry is that the sample is introduced into a vacuum chamber through the inlet and then ionized and advanced through the mass filter or analyser. The ions are sorted by their mass-to-charge (m/e) ratios. After mass analysis, the separated groups of ions are collected and a data system converts the signals from the ions to the desired output. The processes within the MS all occur when under high vacuum conditions.

3.6.4.A IONIZATION CHAMBER

For the analysis of isotopic enrichment, two types of ionization are most widely used, electron impact and chemical ionization.

Electron Impact. Sample molecules enter the ionization chamber where they pass through a beam of electrons. This beam is emitted from a heated filament and is attracted toward a positively charged plate. If a molecule is struck by an electron sufficient energy may be imparted to remove an electron from the sample molecule resulting in a molecular ion:

$$M + e = M^{+} + 2e.$$

The electron trap is held at a potential that is positive with respect to the filament and it is conventional to set the electron beam energy at 70 eV. The energy in excess of that need to ionize the sample is dissipated by fragmentation of the molecular ion. The pattern of fragmentation, as

reflected by the MS, of a sample is consistently characteristic of that particular sample. This ionization mode is more useful for the determination of enrichment at different positions in a given molecule.

Chemical Ionization. A reagent gas is ionized at a relatively high pressure, about 1 Torr, and the resulting reagent gas ions react with sample molecules to yield charged products. Little fragmentation occurs in this type of ionization since little internal energy is imparted to the ion produced in this type of ionization. This method is useful for determining molecular weight or total mass enrichment resulting from stable isotope tracers, without differentiation of the position in the molecule in which the heavy isotope is located.

Other Methods. These are new techniques of ionization which involve desorption i.e the molecule is both evaporated from a surface and ionized. These techniques are of limited application in metabolic research as they have been designed to enable the analysis of larger molecules.

3.6.4.B MASS ANALYSER

Various mass analysers exist, but the ones which are useful in metabolic studies employ either a quadrupole or magnetic mass analyser.

Quadrupole. This mass filter consist of four parallel circular rods that radiate an electrodynamic field. Opposite rods are electrically connected, and the applied voltage consist of a constant DC component and a ratio frequency (RF) component. Because of the oscillating field, a positive ion entering the quadrupole region will oscillate between the electrodes at a specific RF. The quadrupole mass filter will thus allow ions of certain mass to pass through depending on the magnitude of the field.

Magnetic Mass Analyser. This type of analysers is usually used with IRMS instruments. Either a permanent magnet or an electromagnet is used to deflect the molecular ions according to their masses. Each mass is different enough that the major and minor isotopes of the gaseous molecular ions can simultaneously be collected on spatially separate detector plates.

3.6.4.C ION DETECTION

The detection of ions in GCMS work is most frequently enhanced with an electron multiplier. The ions that exit from the mass analyser bombard the surface of the electron multiplier and cause electrons to be ejected from it's

metallic surface and these electrons in turn cause the ejection of more electrons until the signal of each ion is amplified 10^5–10^7 times. Both negative and positive ions can be detected that way. The amount of amplification depends on the energy and mass of the incident ion, the age and condition of the electron multiplier and the voltage applied to electron multiplier.

3.6.4.D DATA HANDLING

Interfacing with a computer is required in order to collect and process the generated data. A computer can be programmed to collect, store and manipulate data in several different ways. For isotopic studies there are two modes that are most useful. One type allows for scanning and storing of several entire spectra. The other is for repeated scanning of only selected ions from a spectrum and is called SIM, which stands for selected or specific ion monitoring.

3.6.5 ISOTOPE RATIO MASS SPECTROMETRY

The IRMS is designed to measure isotopic abundances of elements in gaseous form. Thus when ^{2}H, ^{13}C, ^{15}N, ^{18}O and ^{34}S are used as tracers, their enrichment is most commonly determined from hydrogen and nitrogen gas, carbon dioxide and sulphur dioxide. This instrument is capable of detecting a level of 10 parts per million which is quite adequate for the measurement of $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{18}O/^{16}O$ or $^{15}N/^{14}N$ ratios in metabolic studies.

The inlet system of the MS is connected to the gas holding cells which contain the sample gas and the reference gas. The gas molecules stream from the holding chamber through the molecular leaks into the MS which is under high vacuum. The capillary leak lines lead to a pair of switching valves that admit the sample and the reference gas alternatively to the ion source. All the MS conditions are maintained at the same settings during the analysis of both sample and reference, and if the true ratio of the reference is known, then an absolute ratio for a sample can be determined. Once in the source, the gas molecules are bombarded by electrons that are emitted from the filament wire. Some of the molecules become positively charged and past a series of focusing lenses into the analyser section of the MS. The molecular ions are deflected by the magnet toward the ion detector. The ion detector is usually a Faraday cup or electrometer amplifier. Data handled by a computer or strip chart recorder.

3.6.6 COMPARISON BETWEEN GCMS AND IRMS

The GCMS and IRMS are complementary instruments, and both are useful for performing metabolic tracer studies. The main advantage of the GCMS analysis is the almost limitless variety of samples that can be analysed while small number of pure gases can be analysed by IRMS. Using GCMS is less tedious for any compound other than pure gas and smaller samples are needed than IRMS.

The advantage of the IRMS is the greater precision of measurement of isotopic enrichment. A comparison between GCMS and IRMS is given in table 3.6.1(46,107).

TABLE 3.6.1 Comparison between IRMS and GCMS.

	IRMS	GCMS
Sample type	Pure gas	Volatile organic compound
Sample size	10^{-3}-10^{-6} gm	10^{-7}-10^{-10} gm
Mass analyser	Magnet	Quadrupole
Precision	± 0.0001	± 0.5
Enrichment range	0%-0.5%	0.2%-100%

CHAPTER 7

SMALL INTESTINAL MUCOSAL SAMPLING

3.7.1 INTRODUCTION

Biopsy material from the mucosa of human small intestine free of post mortem autolysis or surgical trauma did not become readily available until 1956 (111). Prior to that small mucosal specimens were obtained by lapratomy or post mortem (112). Later on Crosby and Kugler introduced an intestinal biopsy capsule capable of removing mucosal specimens from any level of the intestinal tract (113). Subsequently many modifications and additional biopsy tubes have been described (114). The peroral small bowel biopsy has altered the concepts of pathological changes and provided an important tool for investigation of certain physiological processes (115).

The introduction of fibreoptic pan-endoscope had undoubtedly revolutionised the examination of the gastrointestinal tract and opened a new chapter in Gastroenterology in the early 1970s (116).

3.7.2 CROSBY KUGLER BIOPSY CAPSULE

This intestinal biopsy capsule obtains samples of mucosa from the small bowel by oral passage of the capsule and attached tubing to the area to be biopsied. Suction created by a syringe pulls mucosa into a port on the side of the capsule and triggers the knife.

3.7.2.A COMPONENTS OF THE CAPSULE

The components of the Crosby Kugler capsule are shown in figure 3.7.1.

These components include; the cylinder which contains two holes, one for obtaining mucosal specimens and the other hole is attached to the polyethylene tubing. The knife is a cylindrical block with a blade on one edge and works by cock-and-trigger mechanism. The knife has a space to fit the screw which secure the spring on assembling the capsule.
The dome is the top of the capsule covering the open end of the cylinder. When the capsule is assembled, a latex rubber diaphragm is used to separate the cylinder and dome. A polyethylene tubing is attached to the cylinder and acts as a transmitter of suction to the capsule as well as serving as the means of retrieving the capsule after taking the biopsy. Other components are, a ring clip to hold the capsule, hypodermic syringe to inject saline and air and to develop suction.

In our studies we used Crosby Kugler capsule, adult model (Quinton, Seattle, Washington 98121, USA) with a diameter of 9.5 mm and a port diameter of 3 mm. The polyethylene tubing we used was 5 feet long, inner diameter of 0.055 inch and outer diameter of 0.075 inch.

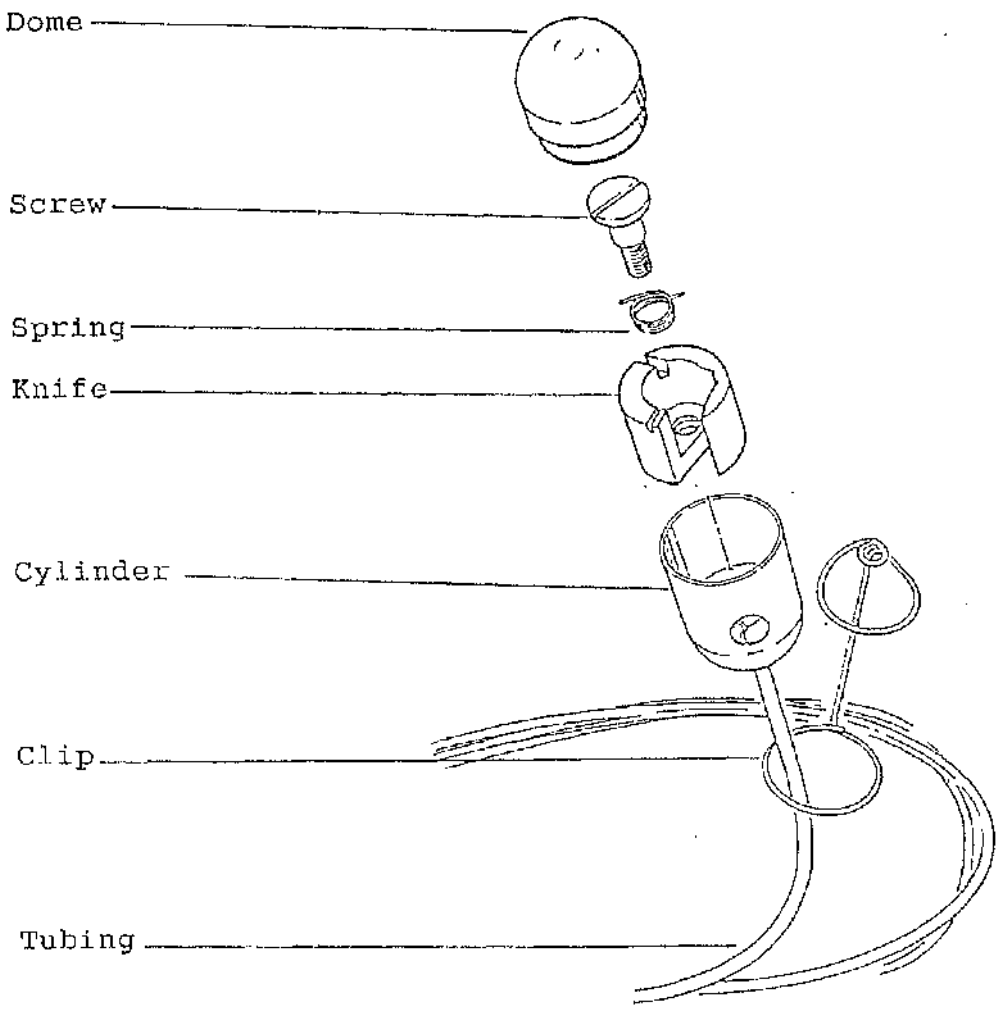


Figure 3.7.1 Components of Crosby Kuglar capsule.

3.7.2.B ASSEMBLING AND PASSING THE CAPSULE

The knife and spring assembly are dropped into the cylinder. The knife is then cocked by turning anti-clockwise and a circular piece of latex rubber diaphragm with a diameter of 2 cm laid across the open end of the capsule. The latex rubber diaphragm converts the dome into air-tight chamber and acts as a gasket binding the dome and the cylinder together. The dome is then pressed into the capsule and secured in it's place with the ring clip. The capsule assembly then checked for leaks or mechanical problems which might prevent in vivo firing. Patient then asked to swallow the capsule with the tubing. A radio-opaque guide wire is threaded into the tube to make it stiffer and help passing the capsule quickly. Once the capsule is in the stomach the position is verified by fluoroscopy. The wire is removed and the capsule is allowed to pass through the pylorus. Sometimes we need to give the patient metoclopramide (Maxolon, Beecham Research, Hertfordshire, England) 10 mg intravenously to speed the passage of the capsule.

3.7.2.C TAKING MUCOSAL BIOPSIES

The capsule is fired when it reaches the desired location which is 10 cm distal to the ligament of Trietz (Figure 3.7.2), verified by fluoroscopy. Normal saline is injected first to clean the tubing, then air injected to clear the saline. The capsule is fired by applying suction with the syringe and creating negative pressure to suck the mucosa inside the capsule and trigger the knife. The capsule is

removed gently, opened and mucosal specimen retrieved. The biopsy is teased onto a piece of mono-filament mesh with the villus side up. A small portion is cut, weighed and sent for dissacharidases analysis. The rest of the specimen is sent for histopathology.

3.7.2.D DISCUSSION

The original design of the capsule has been modified by replacing the original spring with a simple one to simplify it's assembly. The knife block was also modified to accommodate the new coil spring (117).

The use of a guide wire in the polyethylene tubing has many advantages. It assists the passage and positioning of the capsule making the procedure quicker and minimized patient's discomfort (117,118,119,120).

The use of Crosby Kuglar capsule is quite safe, however, occasionally some difficulties might be encountered. The capsule might be slow to progress or difficult to withdraw out through the pylorus. Changing the patient's posture or the use of metoclopramide (Maxolon) and hyoscine-N-butylbromide (Buscopan) usually overcome these problems (119). We have not encountered any of the rare clinical complications with using this capsule, some of these complications are, abdominal pain, pyrexia and gastro-intestinal bleeding. Although small bowel perforation with fatal outcome had been reported (121,122), but this is very rare indeed. Some people found the use of a smaller capsule easier and less unpleasant to swallow by patients (123).

3.7.3 GASTROINTESTINAL ENDOSCOPY

The idea of direct visual examination of the gastrointestinal mucosa dates back to the 1870s, when rigid scopes were used. It was only during the 1960s fibreoptic instruments for direct visual examination of the gut were being developed (124). With the modern scopes most endoscopic examinations are well tolerated and can be performed on an outpatient basis nowadays using only light sedation. The ability to take mucosal tissue specimens is a crucial part of endoscopy. The procedure is carefully explained to the patients and a formal written informed consent is obtained from the patients.

The scopes we used were; forward viewing pan endoscope Olympus GIF XQ 20 for upper gastrointestinal endoscopy and paediatric colonoscope, Olympus PCF for ileoscopy (Key Med Ltd, South end-on-Sea, Essex, England). Patients were sedated with slow intravenous (IV) injection of 5-10 mg midazolam (Hypnovel, Roche Products Limited, Hertfordshire, England). During the procedure oxygen was given via a nasal oxygen cannula (Universal Hospital Supplies LTD, Southgate, London, England) at a rate of 4 l/min. Patients pulse rate and oxygen saturation were monitored during the procedure using pulse oximeter (Ohmeda Biox, BOC Health Care, Louisville, USA).

3.7.3.A MUCOSAL BIOPSY SAMPLING

Full endoscopic examination was performed before taking biopsy to assess the intestinal mucosa. Then the duodenal

mucosa was biopsied distal to the ampula of Vater and the terminal ileum was biopsied within the distal 50 cm from the ileostomy stoma (Figure 3.7.2). The biopsy forceps we used were FB-25 K (Key Med, South end-on-Sea, Essex, England) which is the standard fenestrated type compatible with the instruments we used. Intestinal mucosal samples were dealt with according to the protocol as outlined in chapter 8.

3.7.3.B DISCUSSION

Fibreoptic endoscopy is a safe and well tolerated procedure, however certain precautions need to be observed to avoid potential hazards. These hazards include oversedation, adverse reaction to the drugs used and cardiac arrhythmias particularly in the elderly. Oxygen delivery and vital signs monitoring are essential to avoid these complications. Due care must be paid to the cleaning and disinfection procedures of the scopes to avoid transmission of infections such as salmonella and hepatitis among other infections. Other rare complications such as Perforation, Impaction of the endoscope and bleeding had been reported during advanced therapeutic procedures, under emergency circumstances and in the presence of risk factors such as impaired coagulation and portal hypertension (124).

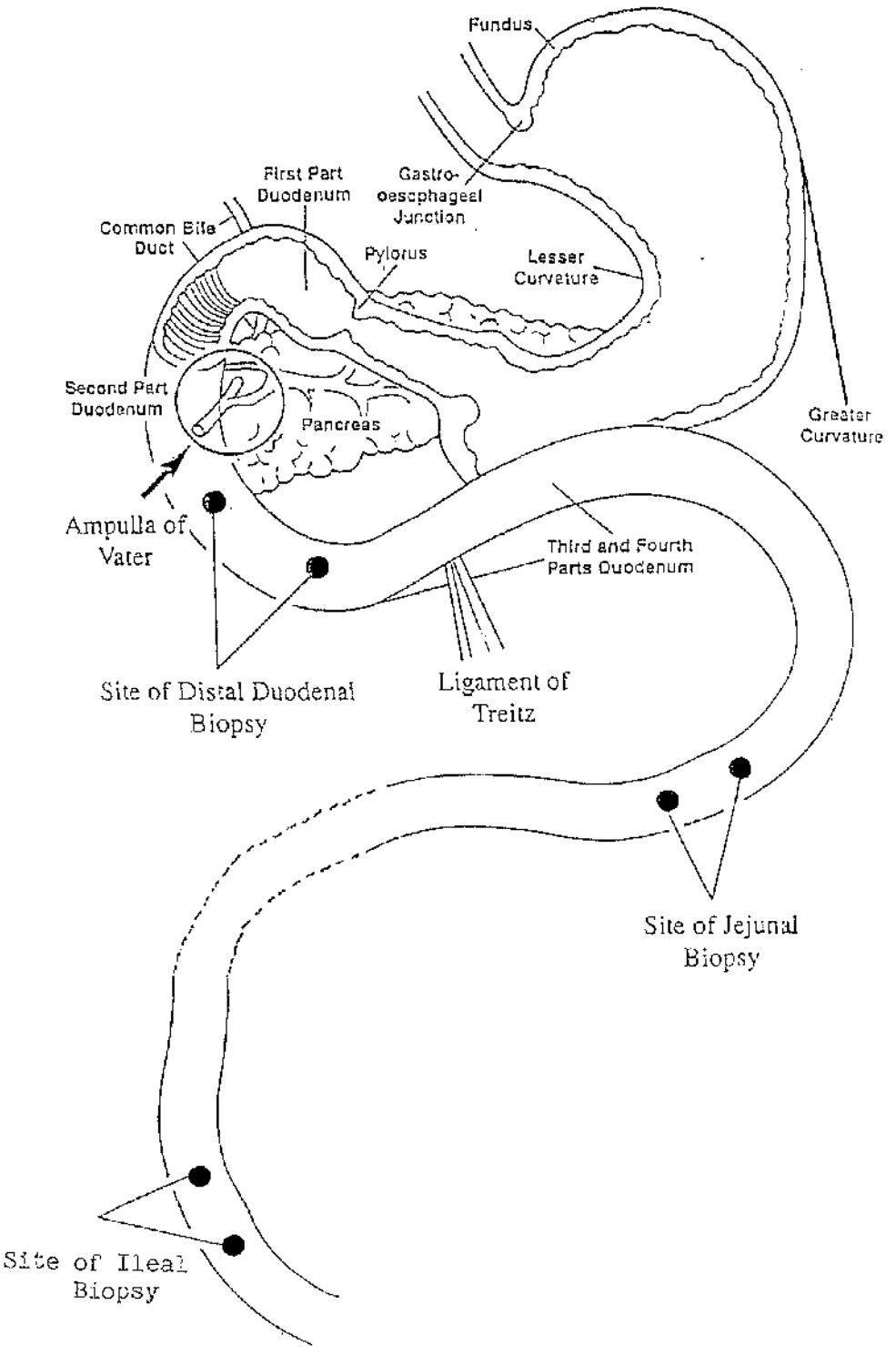


Figure 3.7.2 Diagram of the stomach and the small bowel showing the sites of mucosal biopsies obtained.

3.7.4 HANDLING OF MUCOSAL BIOPSIES

The biopsy specimens were obtained from the small intestinal mucosa and sent for histopathology including histomorphometry, assay of disaccharidases activity and determination of protein and nucleic acids composition as well as the free intracellular and protein bound tracer enrichments.

3.7.4.A *Histopathology.*

Distal duodenal mucosa and terminal ileal mucosa were biopsied using fibreoptic endoscopy. Crosby Kuglar capsule was used to biopsy the jejunal mucosa. Specimens were sent to the pathology department in Buin's solution. The jejunal biopsies were retrieved from the capsule and teased onto a piece of mono-filament mesh with the villus side up, after taking a small portion for disaccharidase activity. The mounted specimens were then dropped in the fixative (Buin's) solution with the villus side down and the mesh up to prevent detachment of the specimen from the mesh. Specimens obtained by biopsy forceps were dropped directly in the fixative solution.

3.7.4.B *Disaccharidases Activity.*

A portion of the jejunal biopsy was weighed quickly and freezed under $-20^{0}C$ for disaccharidase activity assessment. Two distal duodenal biopsies were also taken and dealt with in a similar way. The average weight of these specimens was 10 mg.

3.7.4.C *Protein, Nucleic Acids and Free and Protein Bound Tracer Enrichment.*

Mucosal biopsies from the terminal ileum, distal duodenum and Jejunum were obtained and weighed. They were transported in liquid nitrogen and kept frozen at -70^{0}C until analysis.

CHAPTER 8

DEVELOPMENT OF A TECHNIQUE OF MEASURING MUCOSAL PROTEIN SYNTHESIS

3.8.1 MEASUREMENT OF PROTEIN SYNTHESIS

The methods used to measure protein synthesis in animal and man depend on the fact that proteins are synthesised from free amino acids only. The rate of protein synthesis can be measured as either the rate of incorporation of a labelled amino acid into protein or the decay rate of labelled proteins (37). The methods available now for studying protein turnover (synthesis and breakdown) are, whole-body protein turnover methods (125), methods for regional amino acid metabolism and protein turnover (126) and measurement of protein synthesis by precursor (amino acids tracer) incorporation techniques (127). My work, which is described in this thesis, is concerned with the tracer incorporation techniques to measure the small intestinal mucosal protein synthesis and whole-body protein turnover.

3.8.2 THE PRECURSOR POOL FOR PROTEIN SYNTHESIS

In the 1950s there was a number of attempts to ascertain the source of amino acids for protein synthesis, whether proteins were synthesized from free amino acids or free pre-existing proteins or peptides. It was concluded that proteins were made *de novo* from free amino acids and this conclusion is accepted today (128).

Investigations of amino acids pool in single-cell organisms such as bacteria, yeast and moulds served as a basis for studies on more complex systems (129,130,131,132).

Then different tissues were used for *in vitro* studies such as small muscles (133,134), pancreas (135), foetal bones (136,137), liver slices both from animals (138) and man (139) and submandibular glands (140). There was no adequate means of assessing the viability of the tissue and was difficult to prove the uniformity of the metabolic process and isotope labelling within the tissue as the substrates were supplied by diffusion from the media. A reliable *in vitro* organ culture technique had been developed for small intestine (141). In these studies it is impossible to determine the effect of various metabolic stimuli on the protein turnover. Therefore studies on perfused organs were developed where the circulatory system is intact so that metabolites and tracers can reach the cells by their normal route. Organs perfused in these studies were liver (142,143,144) and heart (145,146).

Experiments in the whole animal *in vivo* have confirmed the results obtained on perfused organs *in vitro*. In this type of studies compartmentation of amino acids in different tissues have been demonstrated (147). In some tissues it has been possible to measure specific activity of the amino acids bound to tRNA which is the real precursor of protein synthesis (148,149,150). The tracers were given via different

routes and methods including continuous feeding (151,152,153), intraluminal perfusion (60,154), intravenous injection (155,156,157) constant intravenous infusion (128,158) and intraperitoneal injection (159).

The use of radioactive tracers in man is not justified particularly with the increasing sensitivity of modern mass spectrometers to measure the stable isotopes enrichment (Chapter 6). Various routes and methods were used to deliver the tracer amino acids in human studies such as; single IV injection, flooding dose IV injection, Continuous IV infusion, Primed Continuous IV infusion, Continuous IG infusion and Primed Continuous IG infusion. Currently, protein synthesis is measured predominantly by using a primed-constant infusion or flooding dose of labelled precursor (46).

3.8.3 CHOICE OF AMINO ACID TRACER

When a labelled amino acid is delivered to the body it is rapidly distributed in the body fluids from which it is incorporated into protein. The method of continuous infusion of labelled amino acid enables rates of protein synthesis to be calculated from the infusion of any amino acid, but a suitable choice will optimize the speed and accuracy of the measurement. A number of factors determine the choice of a tracer amino acid. It should not have metabolic pathways of quantitative importance other than synthesis of protein and oxidation. The time required to reach a plateau enrichment

with a continuous infusion method is short although can be overcome by giving a prime dose. Other factors are not so important nowadays with the availability of sensitive analytical methods. Among these factors is the contamination of the amino acid with the D isomer. The method used to separate the amino acids has an effect as well. Enzymatic method is quicker and cheaper than separation by column chromatography on an automated amino acid analyser although this latter method is more accurate (37). It is immaterial whether the tracer amino acid is essential (127,160) or non-essential (161) as long as the appropriate precursor pool can be identified and the precursor labelling measured (35). It has been shown that similar results obtained using both types of amino acids (162,163).

Leucine has been used as a tracer in many studies because it is rather abundant in protein at about 8-10% (35) and its free pool size is relatively small so it can be rapidly labelled and tracer incorporation into protein will be efficient (164). A general assumption of tracer methodology is that there is no recycling of tracer and any amino acid that enters the protein pool is lost irreversibly in the time frame of a normal tracer experiment (165). It has been claimed that for leucine, recycling of label from protein can be a problem within several hours of tracer infusion (166,167). However, research has shown this not to be the case (165) and it seems safe to assume that there is little tracer recycling in an experiment of normal duration (46).

There have been relatively few studies of the metabolism of valine (168,169,170) compared with studies of leucine metabolism. The ketoacid of valine, alpha-ketoisovalerate (KIV) offers the same technical opportunity to sample intracellular enrichment in a similar way to leucine and alpha-ketoisocaproate (KIC). The rates of plasma flux and oxidation of valine are similar to those of leucine but the transamination equilibrium favours leucine transamination over valine by five fold.

Phenylalanine is not metabolized peripherally and it is virtually entirely metabolized by the liver (46).

3.8.4 CHOICE OF TRACER DELIVERY TECHNIQUE

As mentioned earlier, currently, protein synthesis is measured either by using the *primed-constant infusion* or the *flooding dose injection* technique. In both methods the assumption is that there is a homogeneous metabolic nitrogen pool of which plasma constitutes a part (127,46).

In their study, Halliday and McKeran achieved a plateau labelling of 1[^{15}N]lysine in 14-16 hours (127) therefore to overcome this problem another amino acid with a smaller free pool size, leucine was used and a prime dose was injected before the constant infusion to enable rapid achievement of plateau enrichment of the precursor (160,164). In this method usually small dose of 99% level of labelling used e.g 1 mg/kg prime dose followed by 1 mg/kg/h for 4 h. The use of priming dose was first described in 1954 (171) in conjunction with a constant infusion of tracer. The advantages of reducing the

time to reach isotopic equilibrium are to make it possible to study subjects within a few hours without interfering with patient care and also to avoid the possible changes in the physiological state of the subjects studied if the infusion takes long time, thereby altering the kinetics of the substrate that are being studied. The final equilibrium value is not affected by the prime dose, only the time to reach it is shortened. The ultimate plateau enrichment is the sum of the decline in the enrichment of the prime dose and the rise in enrichment resulting from the continuous infusion. At plateau enrichment (isotopic equilibrium), the rate of appearance of the unlabelled substrate is equal to the rate of tracer infusion divided by the plateau enrichment (46). In this method there is uncertainty in the estimation of the labelling of the precursor pool and difficulty in identifying the true precursor enrichment for the calculation of protein synthesis, hence there is a limit to the accuracy of the calculated values (35,46,164).

The so called flooding dose technique tries to solve the precursor problem by bringing all of the free amino acid pools (intra and extra cellular compartments) including the pool that is used for charging tRNA to a common value of labelling, thus enabelling rates of protein synthesis to be calculated with minimum error (37). A mixture of large dose of tracer amino acid at a relatively low level of labelling (20%) and a tracee amino acid to a total dose of 50 mg/kg body weight is given intravenously over a few minutes. Target tissue is biopsied after about 90 minutes and analysed by

usual methods (172). Theoretically this method should eliminate uncertainty regarding precursor enrichment and it should be possible to use only one plasma enrichment measurement to quantify the true precursor enrichment. Another advantage is the potential for rapid and convenient measurement of protein synthesis rates in human under hospital conditions (46,172). This technique was adapted from the original use of the radioactive tracers (150). On the other hand, there are potential problem with this technique as it assumes that the large dose of the amino acid injected, well in excess of the total body free pools of that amino acid, will have no effect on the rate of protein synthesis. Furthermore, the assumption that there is no delay in the incorporation of tracer amino acid from the free pool to the protein bound pool i.e protein synthesis is instantaneous (46). Using this technique, (172,173,174) the rates of protein synthesis obtained have been approximately twice those obtained with the primed-constant infusion method (175,176). To investigate this, simultaneous measurement of incorporation of continuously infused tracer into protein before and after flooding dose of amino acid was performed. The results suggest that the flooding dose itself may introduce artefacts in the measurement of protein synthetic rate (175,177). Other examples of an apparent increase in the rate of tracer incorporation as a result of flooding-dose protocol can be found for albumin (176), non-albumin protein in blood (35) and for collagen (41). The difference is not attributable to an analysis problem as the Garlick group are

themselves able to reproduce the same values obtained by others using the constant infusion technique. One possible explanation is that the large dose of leucine induces insulin secretion which stimulate protein synthesis and which happens before taking the first blood sample and by that the peak of insulin secretion was missed by Garlick group (164).

3.8.5 THE PRECURSOR PROBLEM

The ideal precursor to measure the protein synthetic rate is the aminoacyl tRNA (35,37). There are difficulties in the measurement of the tRNA. The free tRNA pool is extremely small and the rate of protein synthesis relatively fast, the half time of tRNA pool turnover should be correspondingly small and was calculated to be of the order of seconds (178). Another problem is that direct measurements of the tRNA demand large tissue specimens (1-5 gm) (179) which must be handled with special caution because of the small amount of tRNA present and it's rapid turnover rate (148). Although the plasma KIC labelling is always higher than the intracellular leucin enrichment (46,180), nevertheless, the extent of tRNA labelling appears to be close to that of alpha-KIC which lays between venous leucine labelling and the intracellular leucine labelling (181). It seems now that the labelling in various pools as follow; venous leucine 100%, venous alpha-KIC 82%, leucyl tRNA 78% and intracellular free leucine 72% (182). According to this, any errors in the measurement of the rate of protein synthesis cannot be greater than about 20% (35).

CHAPTER 9

EXPERIMENTAL DESIGN, CALCULATIONS AND STATISTICAL ANALYSIS

EXPERIMENTAL DESIGN

3.9.1 SUBJECTS

Healthy volunteer subjects and patients with various gastrointestinal diseases were recruited during the course of this work. Patients with untreated coeliac disease, alcoholic liver disease and ulcerative colitis treated with total colectomy and ileostomy were included in the studies. Both male and female adults were studied in the post absorptive state i.e asked to fast from 2100 on the evening before the study, which was carried out between 0900 and 1400 of the following morning. There was no adaptation to a standard diet prior to the studies but the subjects and patients ate their accustomed diet. These studies were performed in the Department of Gastroenterology, Glasgow Royal Infirmary University NHS Trust. Each subject gave written consent after a full explanation of the study. All the studies were approved by the local ethics committee of the Royal Infirmary, Glasgow.

3.9.2 TRACERS

The stable isotope tracers L-[1-^{13}C]leucine and L-[1-^{13}C]valine (99 Atom %) were obtained from MassTrace, Woburn, MA. Once weighed according to the protocol and subject's weight, the powder was dissolved in a beaker with the

appropriate amount of sterile, non-pyrogenic 0.9% NaCl solution (150 mmol/l) (Baxter Health Care Ltd, Thetford Norfolk, England), before administration. The solutions were sterilized by passage through a 0.20 micron filter (Acrodisc-DLL, Gelman Sciences, USA).

3.9.3 EXPERIMENTAL PROTOCOL

The patients were studied in the morning following an overnight fast, they neither received food nor drink during the study and remained semi-recumbent in a thermometer environment room temperature (25-27 ^{0}C). Venous blood samples were taken from a 14G/2 mm outer diameter cannula (Venflon-2, Viggo AB, Helsingborg, Sweden) placed retrogradely in an anti-cubital vein and maintained patent by heparin IV flush solution 10 unit/ml (Heplok, Leo Laboratories Limited). Before sampling, 3 ml of venous blood was withdrawn and discarded, then 5 ml of venous blood was withdrawn and centrifuged at 2.8 x 1000 RPM at room temperature for 20 min using IEC Centra-3 centrifuge (Bedfordshire, England). 2 ml of separated plasma was collected and kept in liquid nitrogen at -180^{0}C in 2.0 ml cryogenic vials (Corning, N.Y. 14831). Priming doses of either L-[1-^{13}C]leucine or L-[1-^{13}C]valine 1mg kg^{-1} body weight were given IV over one min in 25 ml of 0.9% NaCl via 0.20 micron filter and using 18G/1.2 mm outer diameter cannula followed by a constant infusion of the same tracer at a rate of 1mg kg^{-1} body weight h^{-1} over 4 h time using 120 ml of 0.9% NaCl (30 ml/h). The infusion was controlled with IVAC-531 volumetric infusion pump (VAC-House,

Harrow, England). Simultaneously the complementary tracer (either L-[1-^{13}C]valine or L-[1-^{13}C]leucine) was given via the enteral route. A priming dose of 1 mg kg^{-1} body weight was dissolved in 50 ml 0.9% NaCl and delivered over two min via a naso-gastric tube, XRO-Paediatric Duodenal tube-39306 (Vygon, Ecouen, France) followed by a constant infusion of the same tracer dissolved in 480 ml of 0.9% NaCl at a rate of 1 mg kg^{-1} body weight h^{-1} for 4 h (120 ml/h) and delivered via a volumetric pump (MHRE-7 Watson - Marlow Limited, Cornwall, England). The position of the naso-gastric tube was checked radiologically by an image intensifier and infusion only started after getting a satisfactory position of the tube. Figures 3.9.1 to 3.9.4 illustrate the experimental protocols followed in the studies.

A group of control subjects and the patients with coeliac disease received two tracer amino acids via the IV and the IG route simultaneously and had an upper gastrointestinal endoscopy to obtain distal duodenal biopsies at the end of the tracer infusions (Figure 3.9.1).

Another group of healthy volunteers received IV L-[1-^{13}C]leucine only, but these subjects had two endoscopies done to obtain intestinal mucosal samples at various times during the tracer infusion. This protocol enabled us to assess the pattern of tracer incorporation into the intestinal mucosa (Figure 3.9.2).

The alcoholic liver disease patients received IV infusion of L-[1-^{13}C]leucine only and had one upper gastrointestinal endoscopy done to obtain duodenal biopsies (Figure 3.9.3).

In the ileostomy group I obtained small intestinal mucosal biopsies from two sites i.e jejunum and ileum at the end of the IV tracer infusion (Figure 3.9.4).

The wet weight of the mucosal biopsies was >50 mg to ensure the availability of adequate amount of tissue for analysis. These samples were immediately frozen in liquid nitrogen, then subsequently stored, with the plasma samples, at $-70^{0}C$ until subjected to analysis. Further biopsies were taken for histology, fixed in Buin's solution, and for dissacharidases assay. Venous blood samples were taken at -15, 0, 60, 120, 180 and 240 minutes respectively and separated plasma kept in liquid nitrogen.

3.9.4 ^{13}C ANALYSIS

Analysis of ^{13}C enrichment in plasma and neutralized perchloric acid (PCA) extracts of freeze-dried, ground mucosal tissue were determined by GCMS. As urea interferes with GCMS, we had to remove it by incubation with Jack-bean urease at $37^{0}C$ for 15 minutes. Plasma amino acids were extracted with methanolic HCl (4:1, v/v), the solvent removed by evaporation, and then the residue redissolved in pyridine before preparation of t-butyldimethylsilyl (t-BDMS) derivatives by reaction with N-methyl-N-(L-BDMS) trifluoro-acetamide (Regis Chemical Company, Morton Grove. IL, USA) (183). GCMS was carried out on Finnigan 1020B instrument (Finnigan MAT Ltd, Hemel Hempstead, UK), fitted with a 20 m x 0.2 mm inner diameter OF WCOT chemically bonded fused silica

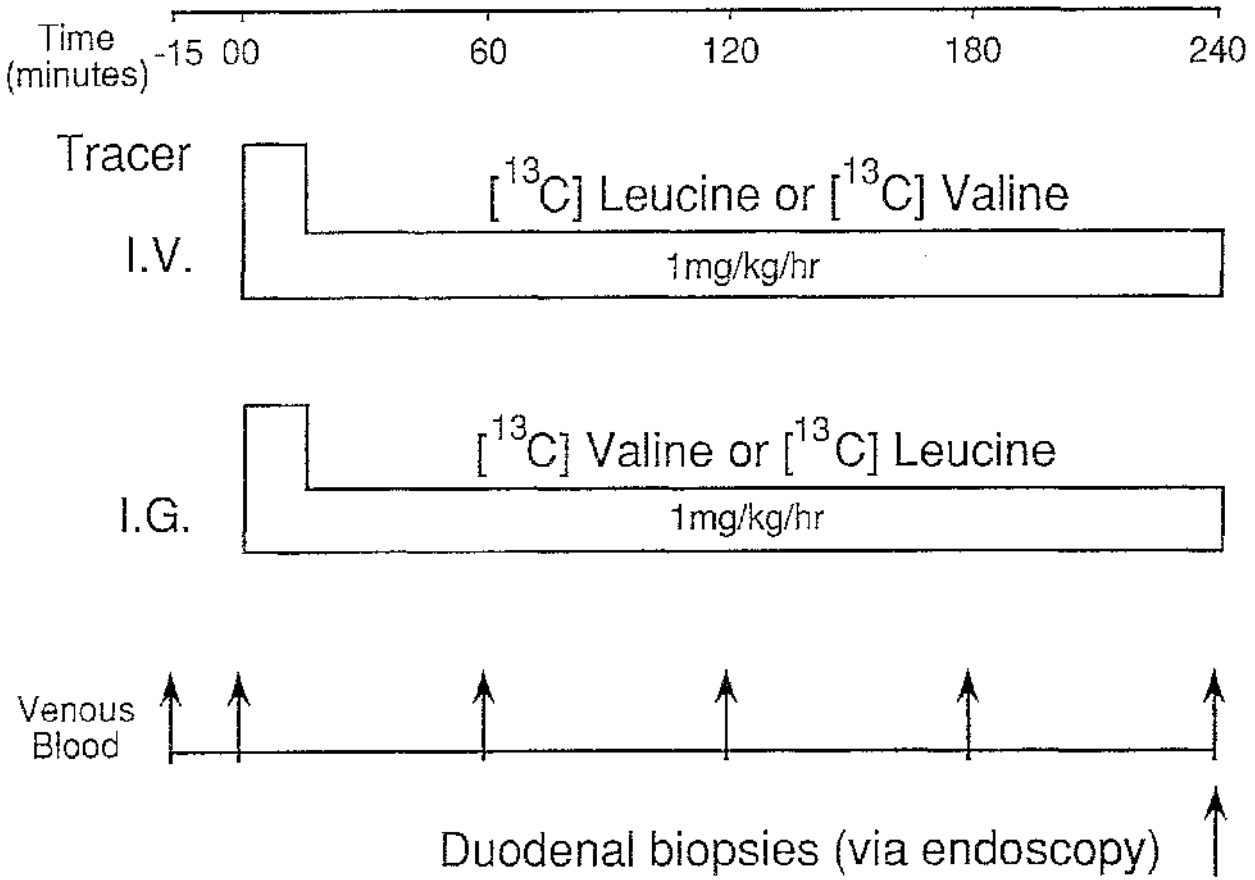


Figure 3.9.1 Experimental protocol of the amino acid infusion studies in normal subjects and coeliac patients.

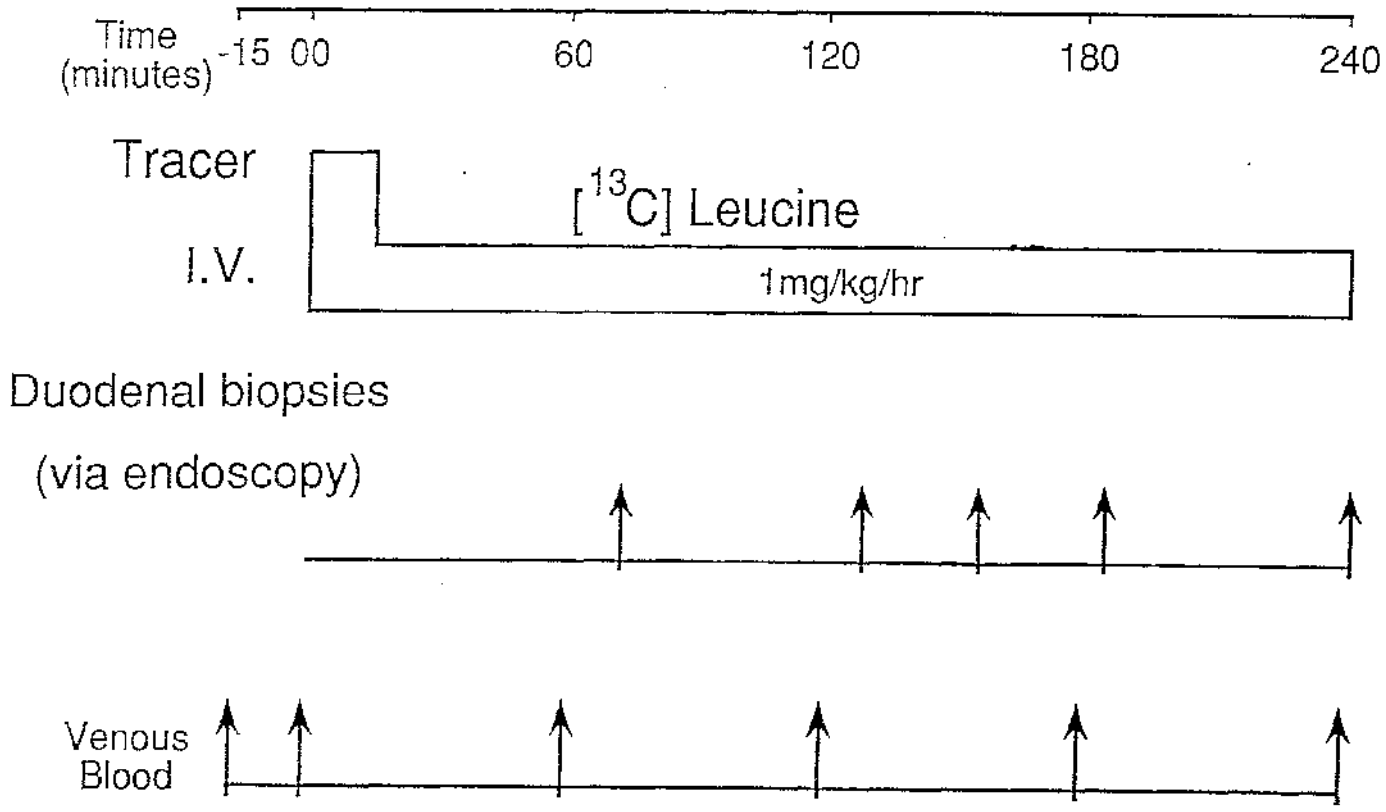


Figure 3.9.2 Experimental protocol of the assessment of the pattern of tracer incorporation into duodenal mucosal protein.

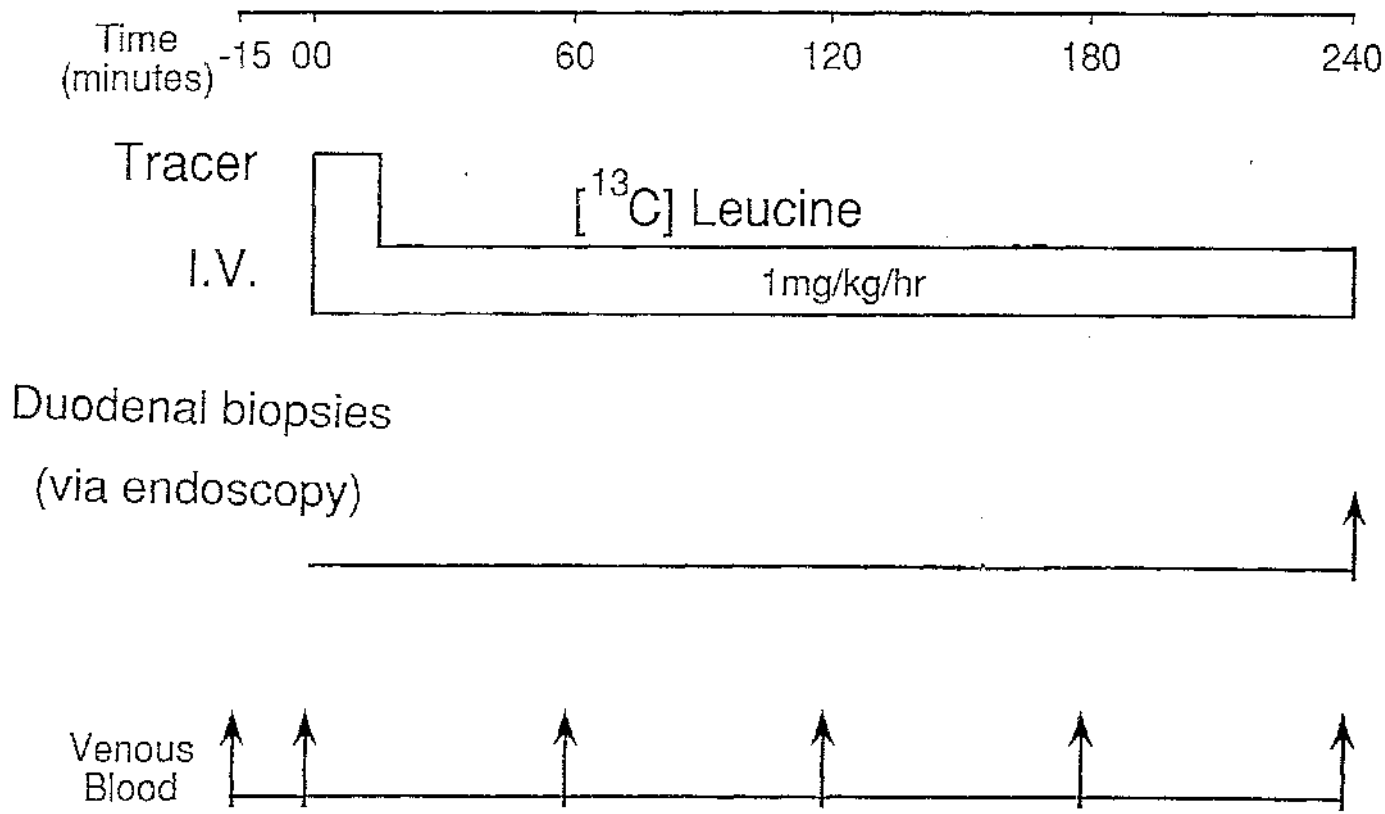


Figure 3.9.3 Experimental protocol of the amino acid infusion study in patients with alcoholic liver disease.

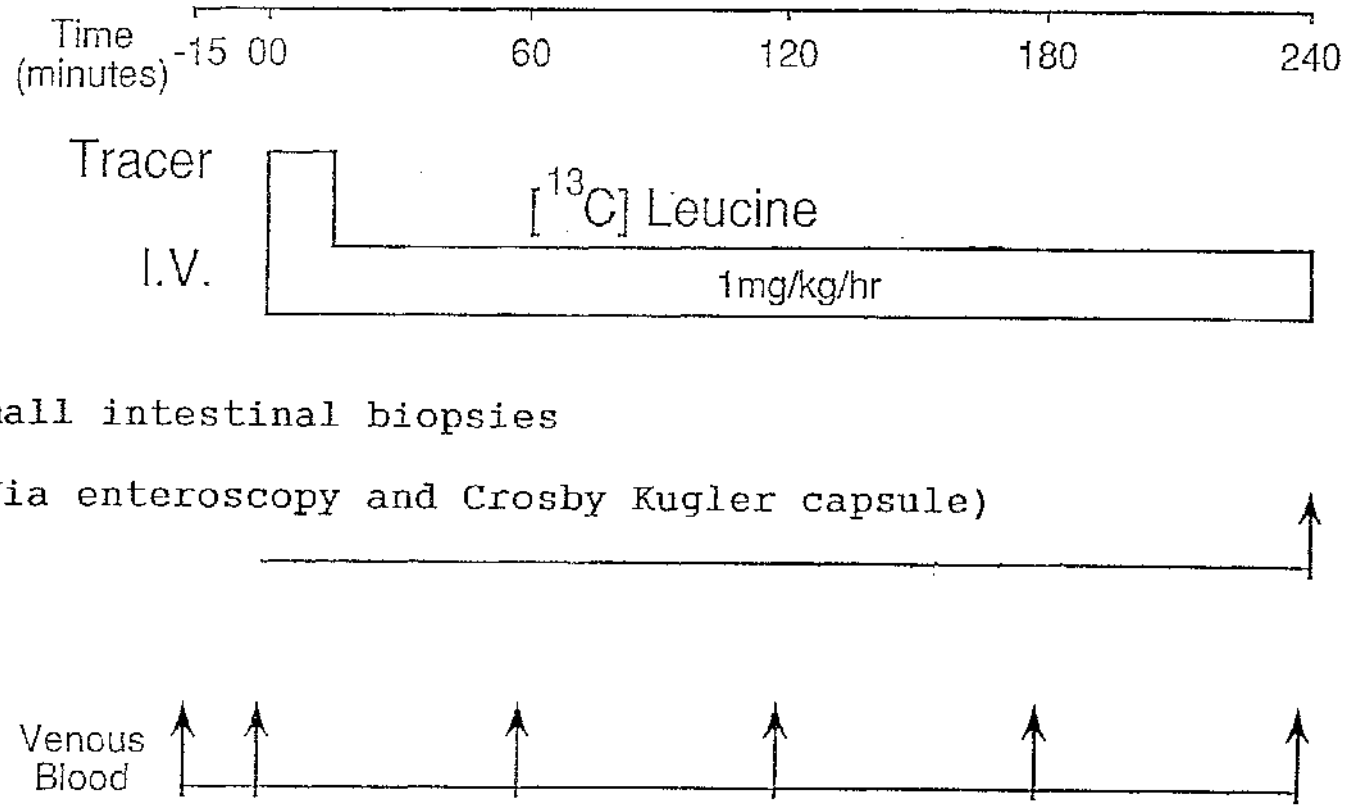


Figure 3.9.4 Experimental protocol of the amino acid infusion study in the ileostomates.

column (Pierce UK Ltd, Cambridge, UK), with temperature programmed from 110 to 270^{0}C at 15^{0}C/min after a 1 min lag. Decane was used as solvent. The injector temperature was 280^{0}C and injection, in the splitless mode, was complete in 30 s. The carrier gas was helium at 50 kPa. The mass spectrometer was operated in the electron impact mode with an ionization energy of 70 eV. The enrichment of [1-^{13}C]leucine and [1-^{13}C]valine were determined by monitoring m/z 302 and 303. The enrichment in ketoisocaproate (KIC) and ketoisovalerate (KIV) were determined by similar methods using *O*-trimethylsilyl quinoxalinol derivatives **(184)** and monitoring m/z 232 and 233.

To determine the ^{13}C enrichment in protein bound leucine and valine, protein from freeze-dried, ground mucosal samples was repeatedly washed with PCA, acid hydrolysed (6M HCl) and amino acids were separated by preparative gas chromatography **(185)**, which is rapid and simpler than the classical ion-exchange chromatography **(186)**. CO_2 was liberated by ninhydrin, which is a powerful oxidizing agent causes oxidative decarboxylation of alpha-amino acids producing CO_2, NH_3 and an aldehyde with one less carbon atom than the parent amino acid **(187)**. The ^{13}C enrichment was determined using the automated breath gas system optimized for small quantities of CO_2 **(188)** and isotope ratio mass spectrometry. We also used the automated ^{15}N ^{13}C analyser (ANCA) Tracer Mass and RoboPrep-G (Europa Scientific Ltd, Crewe, UK). The ANCA system reduced the cost of analysis and improved the

laboratory productivity. It usually needs minimal sample handling as well as smaller samples.

3.9.5 PROTEIN COMPOSITION

The mucosal tissue biopsies were transported and stored in liquid nitrogen or in $-70^{0}C$ freezer prior to analysis. Minimum weight of 50 mg was ground to powder, in liquid nitrogen, and was transferred to a glass tube containing 3 ml of ice cold 0.2 M PCA and centrifuged after 10 min. The free amino acids and ketoacids were extracted in the supernatant. Lipid material then was removed from the pellet in a series of solvent washes. Protein is then solubilised in 0.3 M NaOH in a water bath at $37^{0}C$ for 60 min. An aliquot was removed for the measurement of protein concentration using Folin's reagent as outlined by Lowery (189). The protein then reprecipitated by the addition of 2 ml 1M PCA.

3.9.6 NUCLEIC ACIDS COMPOSITION

RNA was extracted using 0.2M PCA and DNA extracted with 2M PCA at $70^{0}C$ for 60 min. The protein was re-pelleted by centrifugation and hydrolysed by 6M HCl for ^{13}C-analysis. The determination of nucleic acids was by dual wavelength ultraviolet absorption method (190); RNA from UV absorbance of 260 nm and 275 nm, and DNA from UV absorbance at 268 nm and 284 nm, on a UV spectrophotometer using quartz Cuvettes and PCA as a reagent blank.

3.9.7 HISTOLOGY

Intestinal mucosal specimens were fixed in either 10% formalin buffered saline or Buin's solution, embedded in paraffin wax and 4 u sections prepared for light microscopy. Sections were stained with haematoxylin and eosin. Mucosal histology was considered normal when the villous pattern was intact and villus to crypt ratio greater than 2:1 in the absence of any increase in the number of inflammatory cells.

Other methods performed, such as histomorphometry, disaccharidase assay, serum vitamin B_{12} & folate and red cell folate are discussed in the relevant chapters.

CALCULATIONS

The measurement of protein synthesis by the determination of the rate at which a precursor is incorporated is termed the fractional synthetic rate (FSR). This refer to the rate of syntheses, e.g gm/day divided by the total protein pool size, gm. In the steady state, the fractional degradation rate, k_d is equal to FSR (synthesis = breakdown). The value for k_d has no necessary relationship to k_s which is the rate parameter from the amino acid pool to the protein pool and is quite different from FSR. However, the optimal technique to measure the FSR of a specific protein using the primed constant infusion method of an amino acid tracer is to determine the pattern of enrichment of the protein bound amino acid and to use compartmental modeling to calculate the rate parameter relevant to protein turnover. In this case it

is assumed that, if precursor enrichment is constant, then over the first several hours of infusion the tracer will be incorporated into protein in a linear manner. In this case the FSR (k_s, h^{-1}) can be calculated using the expression;

$$k_s\ (\%h^{-1}) = (E_t - E_0/E_p) \times 1/t \times 100$$

where E_t is the enrichment in tissue protein at time t, E_0 is the baseline enrichment and E_p is the enrichment of the precursor (46). The calculation of the FSR by the short-term constant infusion technique has many obvious practical advantages, but is limited by the need to identify the true value of precursor enrichment (E_p) (Chapter 8). The precursor we used in our calculation was the intracellular tracer amino acid enrichment.

Whole-body protein breakdown was calculated using the expression; Protein breakdown (umol of leucin /kg/h) = F (umol/kg/h)/E_{KIC} X 100%, where F is the rate of leucine infusion and E_{KIC} is the enrichment of the ketoacid (191).

STATISTICAL ANALYSIS

Values were expressed as means ± SD and average (range). Statistical analysis by means of Wilcoxon's ranking test for the non parametric, unpaired data (Mann-Whitney test), paired and unpaired Student's t-tests, Spearman's rank correlation or two-tailed t test as appropriate. Differences were considered significant at P values of <0.05.

SECTION IV

MEASUREMENT OF DISTAL DUODENAL MUCOSAL PROTEIN SYNTHESIS IN NORMAL SUBJECTS

CHAPTER 10

GUT MUCOSAL PROTEIN SYNTHESIS MEASURED USING INTRAVENOUS AND INTRAGASTRIC DELIVERY OF STABLE TRACER AMINO ACIDS

4.10.1 INTRODUCTION

In the last fifteen years the use of stable-isotope labelled amino acids has revolutionized the measurement of human tissue protein metabolism studied by estimation of blood tracer flux and incorporation of tracer amino acids into tissue biopsies (46). Although there is a substantial interest in human gastrointestinal protein metabolism and there are reports of measurement of tracer incorporation into normal and diseased gastrointestinal tract tissues (39,192,193,194,195) no robust, well validated method exist for measurement of mucosal protein synthesis in the small intestine in human beings. A question of some significance in designing such a method is whether a preferential route of entry of amino acids to the synthetic precursor pool, via the apical or basolateral membrane, exists in small intestinal mucosal tissue, via the apical or basolateral membrane, both have been supported by work in animal preparations (154,196). Alternatively, the free intracellular pool may simply contain a mixture of basolaterally and luminally derived amino acids from which precursor is drawn according to the degree of mixing (197). Unfortunately no information pertaining to this question exists for human gut *in vivo*.

4.10.2 AIMS

The aims of this study were; 1) to develop a method that would allow measurement of protein synthesis in any gastrointestinal mucosal tissue capable of being sampled either by investigative biopsy or interoperatively and 2) to investigate the effect of route of administration of tracer, whether IV or IG, and 3) to compare the measured rates of distal duodenal mucosal protein synthesis to previously obtained rates in other tissues and the whole body.

4.10.3 SUBJECTS AND METHODS

Seven healthy subjects, six men and one women (age 26 - 59 years, weight 56 - 86 kg) (Table 4.10.1) were studied. All subjects were asked to fast from 2100 on the evening before the study, which was carried out between 0900 and 1400 of the following morning. Before fasting the subjects ate their accustomed diet. Each subject gave written consent, which was carried out under the auspices of the Ethics Committee of the Glasgow Royal Infirmary University NHS Trust. The subjects were investigated over a period of 4 h during which stable-isotope-labelled tracers L-[1-^{13}C]leucine and L-[1-^{13}C]valine were infused, 1 mg.kg^{-1} priming dose followed immediately by constant infusion of 1 mg.kg^{-1}.h^{-1} either IV or IG. In four of the subjects (3 men and 1 women) leucine tracer was given IV and valine tracer IG simultaneously. In the three others (3 men) the routes of delivery were reversed (Figure 3.9.1). There were no differences in body mass index (198) or age in the subgroups. At the end of 4 h of infusion, duodenal mucosa

TABLE 4.10.1 Characteristics of normal subjects studied for duodenal mucosal protein synthesis.

Patient	Age-Y	Sex	Weight-kg	Height-cm	%Ideal BW
1	57	M	65	158	105.01
2	53	M	85	185	112.58
3	59	M	70	178	98.18
4	55	M	56	172	82.23
5	26	M	70	180	96.69
6	51	F	86	160	148.02
7	47	M	80	173	116.45

% ideal BW : % ideal body weight (198)

was sampled distal to the ampulla of Vater via endoscopy, as described before (Chapter 7), to obtain the 50 mg of tissue necessary for analysis. The endoscopic forceps biopsy technique usually required five or six separate samples from different mucosal sites. Venous blood samples were taken before infusion of tracer and at hourly intervals over the period of infusion for the analysis of ^{13}C in leucine, valine, KIC and KIV. Plasma was separated by centrifugation and immediately frozen for later analysis. The labelling of plasma amino acids and keto acids were measured by means of standard GCMS technique using *t*-BDMS derivatives **(199)**. Mucosal tissue samples were frozen in liquid nitrogen immediately upon sampling and were pooled for storage at -70^0C; they were analyzed for concentration of protein, RNA and DNA **(190)**. Total plasma protein from the preinfusion samples [precipitated by use of 10% (wt/vol) PCA] was used to estimate basal body protein ^{13}C labelling for use in the calculation of mucosal protein synthetic rate. In our experience the background enrichment of human tissue proteins from whatever tissue source varies by very little. The average basal value for leucine was 17 ± $1\%_0$ over Pee Dee Belamnite (PDB) and for valine 23 ± $1\%_0$ over PDB. The labelling with ^{13}C of leucine and valine in hydrolysed protein using preparative GC and IRMS of the CO_2 liberated by ninhydrin **(185)**. Values for labelling were expressed as atom or mole percent excess as appropriate, and rates of protein synthesis were calculated as described in chapter 9. The increase in the enrichment in tissue protein over the basal

value at time t was measured from the actual value of labelling in the mucosal protein obtained after 1-4 h of infusion which was 250-1,000 $\%_0$, depending on the rate of tracer delivery.

Statistical significance of differences was assessed by means of paired and unpaired Student's t-test; significance was assigned at the 5% level.

4.10.4 RESULTS

Mean values and their standard errors are presented. During IV and IG infusion of [^{13}C]leucine and [^{13}C]valine the plasma tracer labelling attained plateau values which were maintained within 12% for 4 hrs. The plateau values of the IV tracer were 5.46 ± 0.25 APE (atom percent excess), 10% higher than the plateau values of the same tracer given IG, 4.99 ± 0.25 APE, IV vs. IG, (paired t-test) $P < 0.01$ (Figure 4.10.1).

The route of tracer administration strongly influenced the gradient of labelling between the leucine and valine in the intracellular water and in the plasma; for IV tracer the plasma to intracellular amino acid pool ratio was 1.73 ± 0.16 but for IG leucine or valine the ratio was reversed 0.65 ± 0.12, $P < 0.002$ (Figure 4.10.2).

IG infusion resulted in the attainment of a higher rate of labelling of the intracellular mucosal amino acid pool than IV infusion with leucine or valine (Figure 4.10.3).

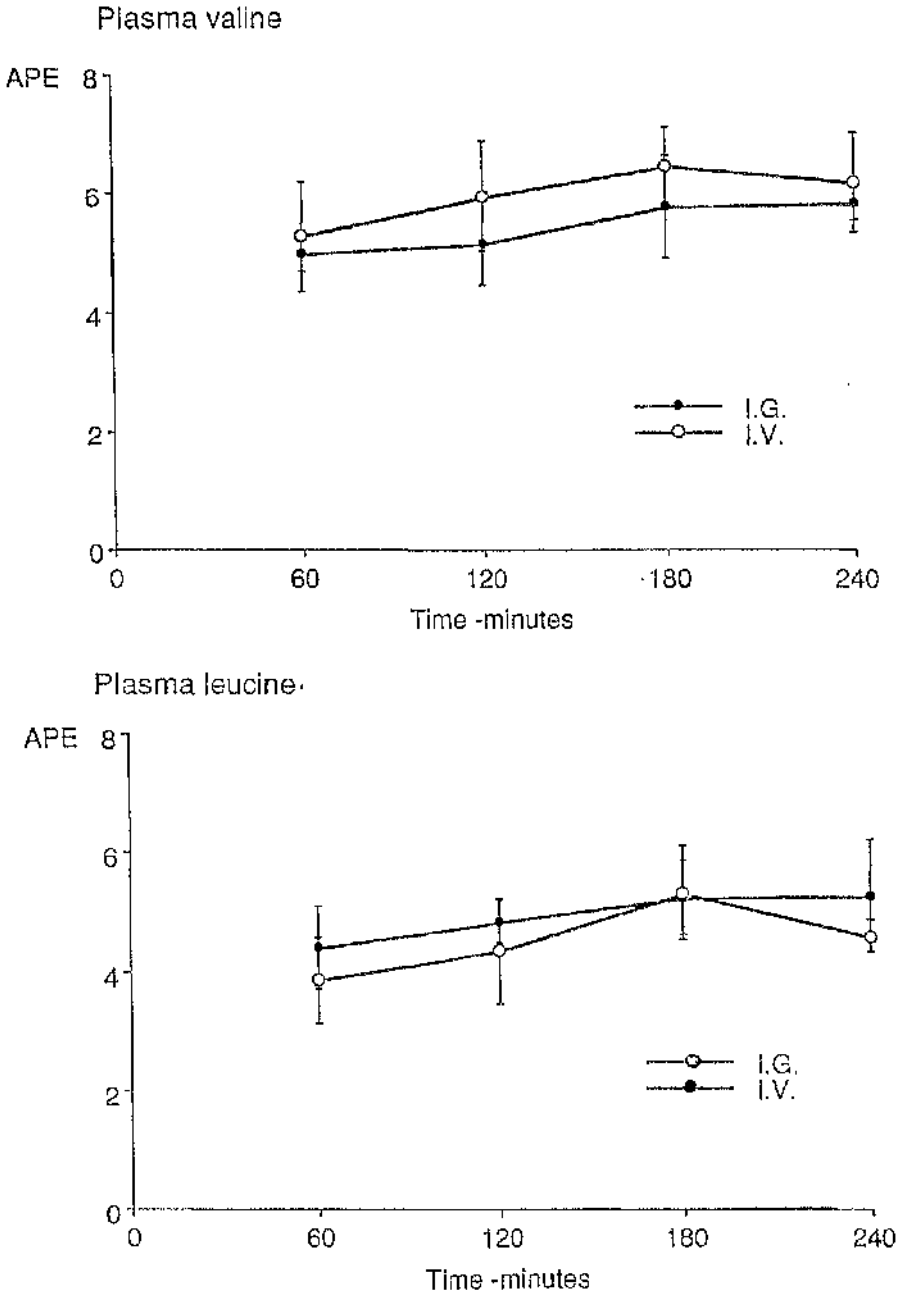


Figure 4.10.1 Plasma labelling of valine and leucine tracers.

The extent of incorporation of tracer leucine and valine into duodenal mucosal protein was 2.3 - 3 folds higher after 4 h of infusion of tracer given IG than when tracer was given IV (Figure 4.10.4).

However, when the rates of incorporation were related to the extent of labelling of the appropriate tracer in the intracellular mucosal water, the rate of protein synthesis in the distal duodenal mucosa became indistinguishable at about 2.5% h^{-1}. Exact rates were 2.58 ± 0.32% h^{-1} for IV administration of tracer and 2.45 ± 0.36% h^{-1} for IG administration of tracer (Figure 4.10.5).

If the plasma keto acids of leucine and valine obtained during IV infusion were used as surrogate indices of the intracellular labelling the protein synthetic rate calculated were 1.8 ± 0.4% h^{-1}, a value not significantly different from that observed using the mucosal free labelling values (Figure 4.10.6).

The rate of labelling of the respective keto and amino acids with ^{13}C also depended upon the route of administration. When the tracer was given via the IG route the mean keto acid/amino acid labelling ratio was 0.94 ± 0.02 for alpha-KIC and leucine and 0.93 ± 0.02 for alpha-KIV and valine, whereas the respective ratios for the IV routes were 0.76 ± 0.07 and 0.78 ± 0.02 (Figure 4.10.7).

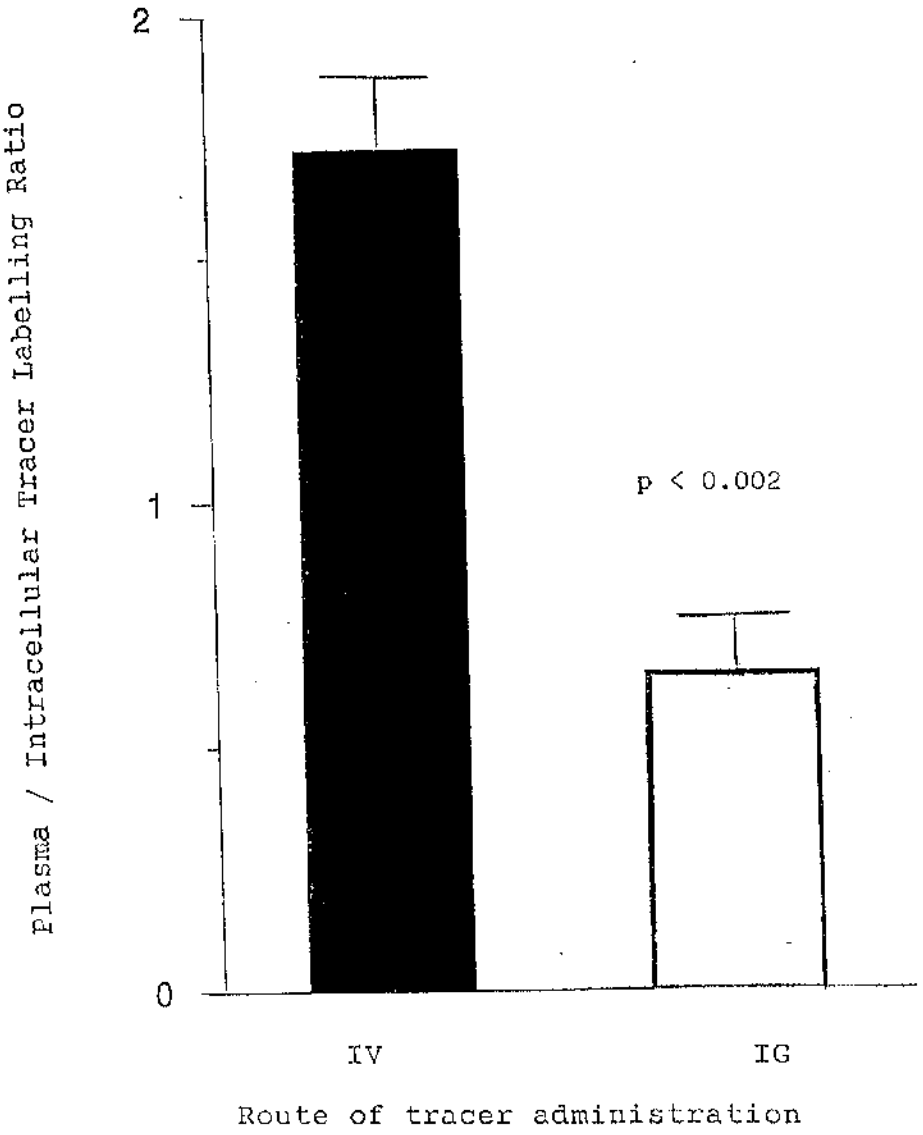


Figure 4.10.2 Plasma/intracellular gradient of tracer labelling for IV and IG routes.

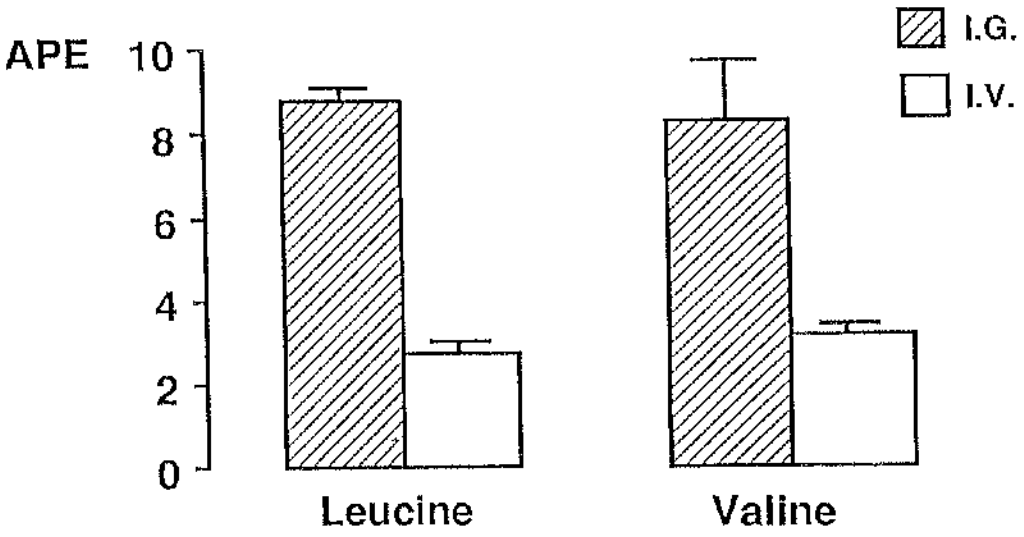


Figure 4.10.3 Intestinal mucosal free tracer enrichment.

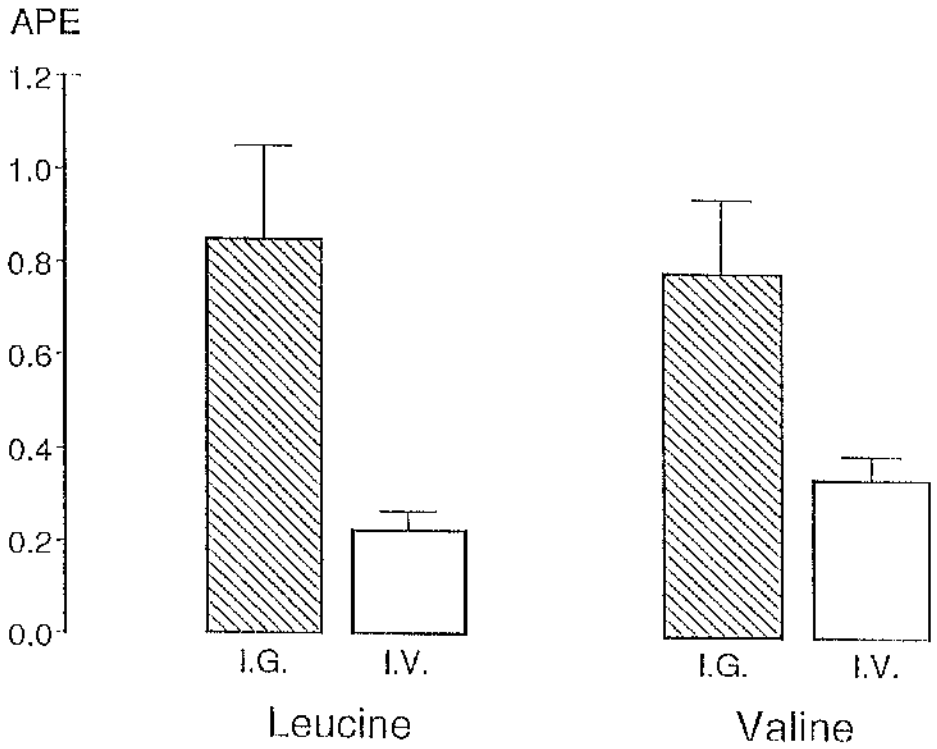


Figure 4.10.4 Intestinal mucosal protein bound tracer enrichment.

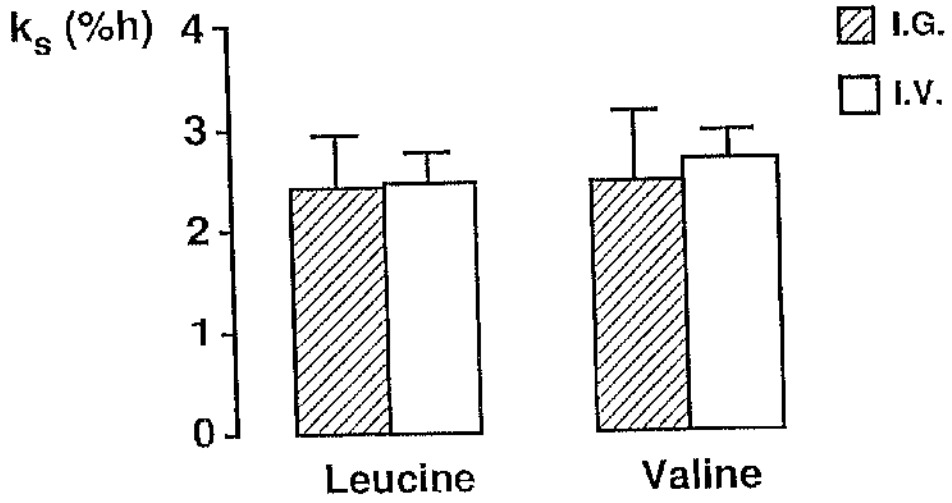


Figure 4.10.5 The rate of intestinal mucosal protein synthesis.

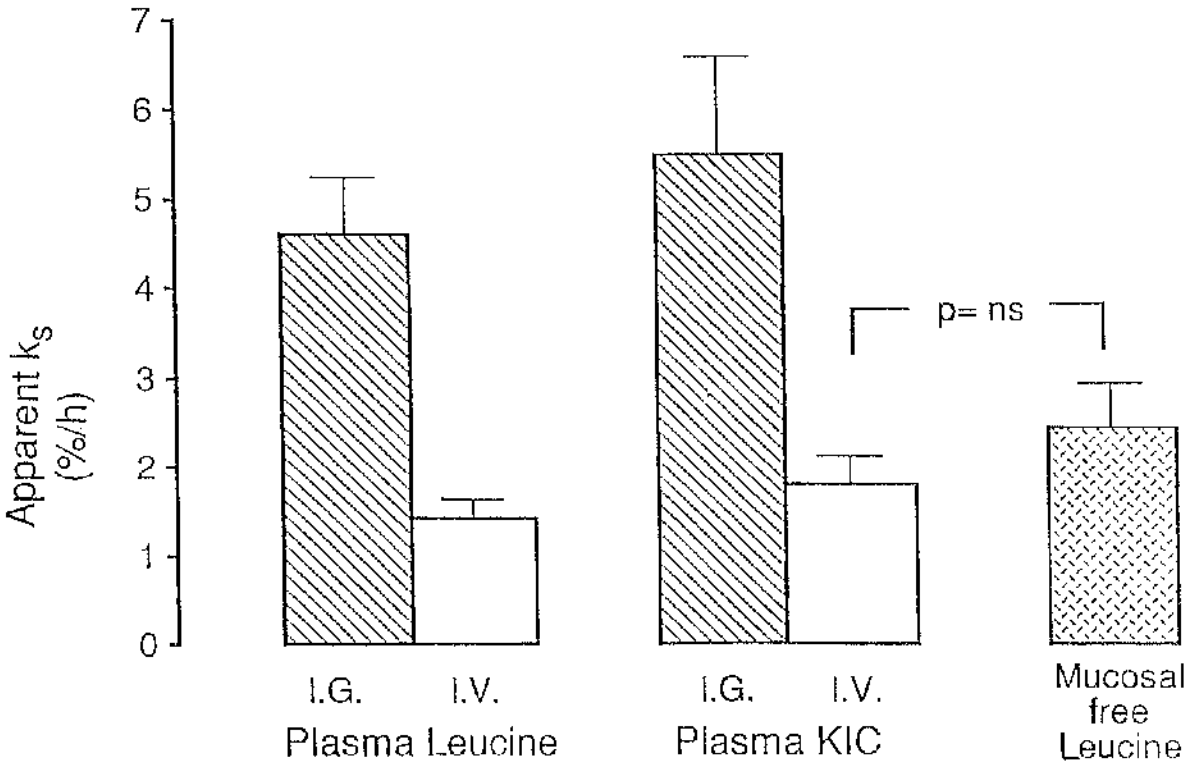


Figure 4.10.6 Which plasma precursor best represent the intramucosal precursor?

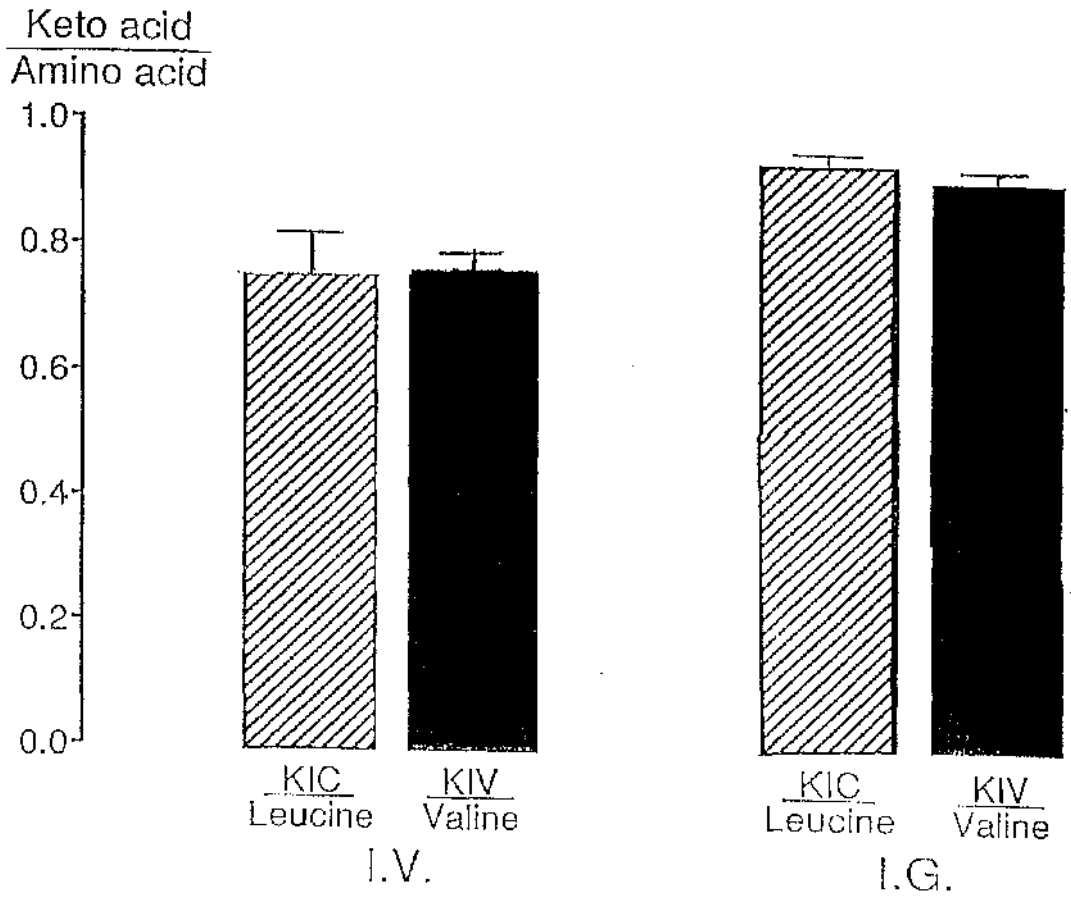


Figure 4.10.7 Keto acid/amino acid labelling ratios

Mucosal tissue showed a high concentration of RNA relative either to DNA or to protein, indicative of a substantial capacity for protein synthesis (Table 4.10.2). The rates of protein synthesis expressed in terms of RNA were 0.32 protein.h^{-1}.mg RNA^{-1}.

TABLE 4.10.2 Mucosal tissue composition and protein synthetic capacity.

Protein $mg.g^{-1}$	DNA $mg.g^{-1}$	RNA $mg.g^{-1}$	Prot. synt. capacity mg $prot.h^{-1}$.mg RNA^{-1}
15.24 ± 0.30	0.68 ± 0.04	1.20 ± 0.06	0.32 ± 0.04

4.10.5 DISCUSSION

The observed higher rate of labelling of the mucosal intracellular free amino acid pool with tracer amino acids after intragastric infusion was not unexpected given the fact that the apical membrane of mucosal cells contain concentrative Na-dependent amino acid carriers for the branched chain amino acids (BCAA) whereas the basolateral membrane contains only non-concentrative facilitated diffusive carriers (200). Nevertheless, despite the substantial differences in the extent of labelling of the intracellular and plasma pool of leucine and valine when the tracers were administered IG or IV, and despite the substantial differences in the absolute extent of labelling

of mucosal protein, the rate of mucosal protein synthesis calculated on the basis of the intracellular amino acid pool labelling were identical.

No information is available regarding the relationship between the various free amino acids pools and the labelling of leucyl or valyl tRNA in duodenal mucosal protein. We have previously found that in the pig, leucyl tRNA in stomach mucosa is labelled to an extent which is indistinguishable from that of alpha-KIC during primed constant infusion of [^{13}C]leucine (201). It may therefore be that calculating protein synthesis based on the intracellular amino acid labelling (which is probably lower than the aminoacyl tRNA) slightly overestimates the true duodenal mucosal protein synthesis and that the true rate lies somewhere between a rate calculated using plasma alpha-keto acid (for IV infusion only) and the intracellular values.

The results suggest that in the post-absorptive state, there is no detectable preferential channelling of amino acids into protein synthesis from the apical or basolateral membrane of the small intestinal mucosal cells. The question of whether or not such channelling occurs has been controversial, with experimental findings favouring each hypothesis (197,154). However, the present results, apparently the first obtained from physiological kinetic studies in human beings *in vivo*, tend to support the idea that the free intramucosal pool obtains amino acids from a variety of sources and that as expected from previous work on rats (196), neither the basolateral nor the apical route is

favoured. Even so, the question may have a different answer if asked for the fed state in which the availability of luminal amino acids will be greater, and it would be unwise to extrapolate from the present findings to other nutritional states.

The lower plateau values for plasma labelling for the tracers infused via the IG route than the IV route suggested that the IG tracers were being diluted to a greater extent by amino acids released from protein breakdown, most likely in the splanchnic tissue. Such an interpretation was first applied to similar findings obtained under similar circumstances by Hoerr and colleagues (202).

The relative rates of labelling of the keto and amino acids for the tracers used agrees with the results obtained previously by Hoerr et al (202). The results suggest that there is little transamination of the BCAAs during their passage from the gastrointestinal lumen into the peripheral blood in accordance with the known low capacities of the gut and liver for BCAA transamination (203).

The fractional rates of protein synthesis observed in the duodenal mucosa were high, being some 25-50 times greater than those previously observed for muscle (175,204) and also about two to three times greater than those determined for normal colonic mucosa (39,173,194). It is impossible to exactly quantify the total amount of small intestinal mucosal protein in order to calculate the absolute amount of protein synthesis contributed by the gastrointestinal tract but the high rate suggests that mucosal protein synthesis is a major

contributor to whole body protein turnover. The extent to which human mucosal protein synthesis can be modulated by physiological influences including nervous, hormonal and nutritional effects, remains to be determined.

4.10.6 SUMMARY

We measured the rates of mucosal protein synthesis during the simultaneous delivery of [1-^{13}C]leucine and [1-^{13}C]valine delivered either IV or IG to investigate any influence of the route of supply of the tracers. Depending on the route, there was marked differences in the gradient of labelling between the plasma and intramucosal leucine and valine i.e, for IV tracers the ratio was 1.73 ± 0.16, but for IG tracers it was 0.65 ± 0.12 ($p < 0.05$). The calculated fractional rates of protein synthesis were identical when based on the intracellular labelling of the leucin or valine tracer i.e, with IV 2.58 ± 0.32 %/h and with IG 2.45 ± 0.36 %/h. The results demonstrate that a robust and reproducible method of measurement of gastrointestinal mucosal protein synthesis has been developed and that use of either IV or IG routes of tracer administration gives comparable results. The high rates measured suggest that the gastrointestinal mucosa contributes substantially to whole body protein synthesis in normal healthy subjects.

CHAPTER 11

THE PATTERN OF TRACER AMINO ACID INCORPORATION INTO INTESTINAL MUCOSAL PROTEIN

4.11.1 INTRODUCTION

The mathematical approaches for measuring the incorporation of labelled amino acid, and release of labelled amino acid from, individual protein in body cells or tissue follow established precursor-product relationship (37). The measurement of protein synthesis by the determination of the rate at which a precursor is incorporated in a specific protein is currently made by using a primed-constant infusion or a flooding dose of labelled precursor (Chapter 8). Synthesis of a specific protein is termed the FSR (Chapter 9) which is, in traditional modeling is not necessary related to the k_s even in the steady state, although the k_d is equal to FSR (protein synthesis = protein breakdown). However, the value of k_s is equal to FSR if the pattern of incorporation of the precursor in the protein is linear (46).

4.11.2 AIM

The aim of this study was to check whether the rate of incorporation of tracer into small bowel mucosal protein was linear in order to validate the method we used to calculate protein synthetic rate.

4.11.3 SUBJECTS AND METHODS

Four healthy subjects 2 men and 2 women (Table 4.11.1) were studied. They were infused with IV [1-^{13}C]leucine only. In order to measure the enrichment of the label in the mucosal tissue we needed to take mucosal samples at various points during the 4 h infusion. Each subject had two endoscopic procedures and distal duodenal biopsies were obtained using biopsy forceps, taking 5-6 separate samples from different mucosal sites. Biopsies were taken either at 1, 2, 2.5, 3 h of infusion, and also at 4 h of infusion. Blood samples were taken before infusion of tracer and at hourly intervals over the period of infusion for the analysis of ^{13}C enrichment (Figure 3.9.2). Plasma was separated by centrifugation and immediately frozen with the mucosal tissue samples for later analysis. Standard GCMS techniques were used to measure ^{13}C plasma enrichment. The labelling with ^{13}C of leucine in mucosal protein using preparative GC and IRMS.

TABLE 4.11.1 Characteristics of normal subjects studied for the pattern of duodenal mucosal tracer incorporation.

Patient	Age-Y	Sex	Weight-kg	Height-cm	%Ideal BW
1	54	M	87	185	115.23
2	24	F	63	166	102.77
3	21	M	62	162	97.95
4	40	F	79	160	135.97

4.11.4 RESULTS

During IV infusion of [1-^{13}C]leucine the plasma leucine labelling attained a plateau value of 5.5 ± 0.35 APE (mean ± SE); this level of labelling was reached within half an hour of the beginning of our infusion protocol. The slopes of the lines for the [1-^{13}C]leucine incorporation into duodenal mucosal protein showed a variance of no more than ± 15%, $y = 0.050x + 0.044$, $r^2 = 0.88$ $P < 0.01$ (Figure 4.11.1), suggesting that the rate of incorporation was linear with time. Time zero values in the figure were obtained from basal plasma protein enrichment. Lines show individual regressions for 4 subjects for 3 values obtained. The average rate of mucosal protein synthesis calculated in these subjects on the basis of the intracellular mucosal leucine labelling was 2.6 ± 0.38 $\%.h^{-1}$.

4.11.5 DISCUSSION

The results of this study indicate that leucine tracer was incorporated into gastrointestinal protein to an extent proportional to the time of exposure of the tissue to the tracer at least when delivered IV. We have assumed that this is also the case for the tracers delivered IG. Any error introduced by this assumption is likely to be small given the convergence of the values obtained for the rate of protein synthesis based on the free intracellular tracer amino acid labelling. Therefore the k_s calculated in this infusion protocol is equal to the FSR when using the expression K_s $(\%h^{-1}) = (E_t - E_0/E_p)$ X $1/t$ X 100.

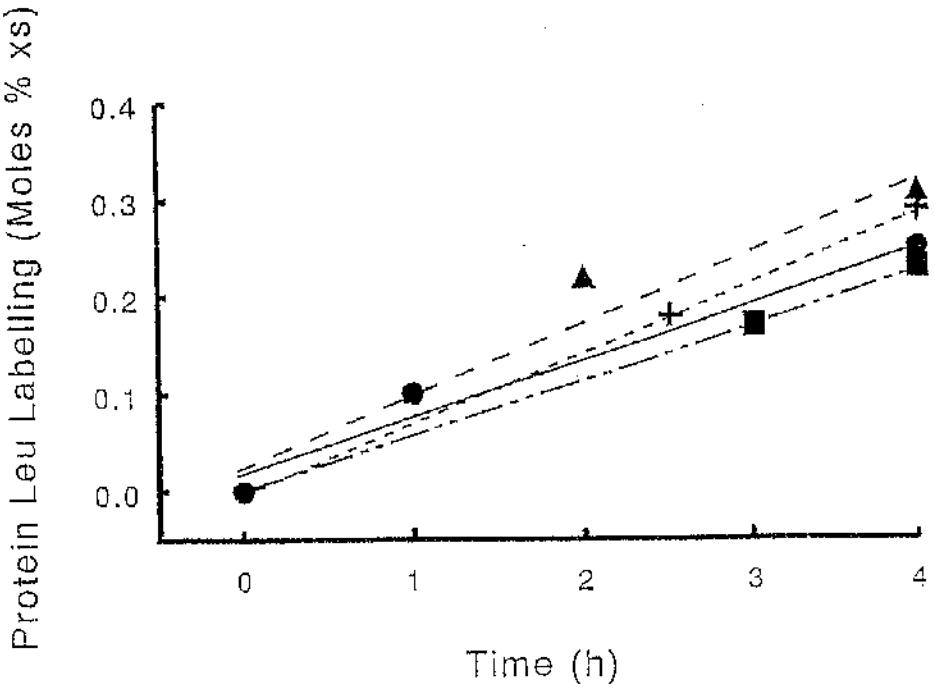


Figure 4.11.1 Leucine incorporation into duodenal mucosal protein over time.

4.11.6 SUMMARY

We measured the rate of mucosal protein synthesis and the rate of tracer incorporation in the duodenal mucosal protein during IV delivery of [1-^{13}C]leucine. Tracer incorporation into mucosal protein was linear with time during a steady state when the plasma tracer enrichment reached a plateau value. This linear incorporation of tracer amino acid into the mucosal protein validates the method we used to calculate the FSR.

SECTION V

APPLICATION OF THE TECHNIQUE OF SMALL INTESTINAL MUCOSAL PROTEIN SYNTHESIS IN COELIAC DISEASE

CHAPTER 12

ELEVATED RATES OF DUODENAL MUCOSAL PROTEIN SYNTHESIS *IN VIVO* IN PATIENTS WITH UNTREATED COELIAC DISEASE

5.12.1 INTRODUCTION

Coeliac disease (gluten-sensitive enteropathy, non-tropical sprue) is characterized by: generalized malabsorption; a typical, but nonspecific, small intestinal mucosal lesion; and a clinical and slower histological response to withdrawal of gluten-containing foods from the diet. The hallmark of coeliac disease is subtotal villous atrophy, abnormal epithelial cells on the mucosal surface and increased cellularity of the lamina propria (205). Rates of epithelial cell renewal and migration in coeliac disease have been reported to be increased six fold and the epithelial cells lining these crypts contain numerous mitotic figures (206,207).

We have developed a safe, reproducible and reliable method to measure protein synthesis in the gastrointestinal mucosa using branched chain amino acids labelled with ^{13}C (Chapter 10) and used it to measure the rates of protein synthesis in the duodenal mucosa in patients with coeliac disease. No measurements of protein synthesis have previously been carried out *in vivo* in coeliac patients.

5.12.2 AIMS

The main aims of this study were; 1) to measure the rate of small intestinal mucosal protein synthesis in untreated coeliac patients when the tracer was administered by IG and IV routes, 2) to compare the results with those results obtained from normal subjects, and 3) to determine the effect of route of delivery on the rate of protein synthesis in coeliac patients.

5.12.3 PATIENTS AND METHODS

Eight patients (51 ± 10 y, 57 ± 11 kg, 160 ± 6 cm) with newly diagnosed untreated coeliac disease, four men and four women (Table 5.12.1) were studied. Each patient gave written consent after a full explanation of the study. Approval for the studies was obtained from the local Ethics Committee of Glasgow Royal Infirmary.

The criteria for diagnosis of coeliac disease were; clinical history of chronic diarrhoea with or without the passage of watery, pale stools with abdominal discomfort, distension and weight loss; subtotal villous atrophy and inflammatory cellular infiltration of the lamina propria, mainly plasma cells and lymphocytes, in all patients as shown on jejunal biopsies obtained by the Crosby-Kugler capsule **(113)**; clinical and histological response to the introduction of a gluten free diet. None of the subjects studied were taking any additional medication such as non-steroidal anti-inflammatory analgesics.

TABLE 5.12.1 Characteristics of coeliac patients studied for duodenal mucosal protein synthesis.

Patient	Age-Y	Sex	Weight-kg	Height-cm	%Ideal BW
1	45	F	40	151	74.63
2	65	F	64	160	110.15
3	52	F	52	158	91.23
4	52	M	45	158	72.70
5	39	M	64	167	97.71
6	65	F	67	159	116.52
7	51	M	67	169	100.6
8	40	M	65	159	104.50

% ideal BW : % ideal body weight (198)

L-[1-^{13}C]leucine and L-[1-^{13}C]valine both 99 Atom %, immediately before administration, were dissolved in sterile, non-pyrogenic 0.9% NaCl solution and were sterilized by passage through a 0.20 micron filter.

The patients were studied in the morning after an overnight fast. Venous blood samples were taken, centrifuged and separated plasma was kept in liquid nitrogen. Priming doses of either L-[1-^{13}C]leucine or L-[1-^{13}C]valine (1mg/kg body weight) were given IV over one min; a constant infusion of the same tracers at a rate of 1mg /kg body weight/h using 120 ml of 0.9% NaCl was then continued for 4 h.

Simultaneously, the other tracer (either L-[1-^{13}C]valine or L-[1-^{13}C]leucine) was given via the enteral route. A priming dose of 1 mg/kg body weight in 50 ml 0.9% NaCl was delivered via a naso-gastric tube over two min followed by a 4 h constant infusion at 1 mg/kg body weight / h in 480 ml of 0.9% NaCl (120 ml/h). After 240 min of continuous infusion, upper gastrointestinal endoscopy was performed and multiple distal duodenal biopsies were obtained. The range of the pooled wet weight of biopsies was 58.0-65.1 mg. Further biopsies were taken for histology and disaccharidase assay. Venous blood samples were taken at -15, 0, 60, 120, 180 and 240 min respectively and separated plasma kept in liquid nitrogen with the biopsies (Figure 3.9.1).

Biopsy tissue was quickly weighed and transferred for storage at -70^{0}C until disaccharidase estimates were done. Before assay the biopsy tissue was homogenized in chilled distilled water and centrifuged. The disaccharidase

activities (lactase, maltase and sucrase) were assayed in the supernatant by a modification of the Dahlqvist method (208).

The labelling of plasma leucine, valine, alpha-KIC and alpha-KIV were measured by means of standard GCMS techniques using t-BDMS derivatives (199). Mucosal tissue samples were frozen in liquid nitrogen immediately on sampling, and samples pooled for storage at -70^0C. The labelling with ^{13}C of leucine and valine in hydrolysed protein was done by using preparative gas chromatography and IRMS of the CO_2 liberated by ninhydrin as previously described (185). Plasma protein from the pre-infusion samples was used to estimate basal body protein ^{13}C labelling for use in the calculation of mucosal protein synthetic rate. The pooled mucosal tissue samples were analysed for concentration of protein, RNA, and DNA (189,190).

We calculated protein synthesis as fractional rate using the equation k_s ($\%h^{-1}$) = $(E_t - E_0/E_p)$ x $1/t$ x 100. The precursor we used in our calculation was the intracellular tracer amino acid. The calculation by this equation depends on the fact that the tracer incorporation into the protein is linear, which we have previously demonstrated (Chapter 11).

Whole-body protein breakdown was calculated using the expression; Protein breakdown umol of leucine /kg/h = F (umol/kg/h)/E_{KIC} x 100%, where F is the rate of leucine infusion and E_{KIC} is the enrichment of the keto acid (191).

Values were expressed as means ± SD. Statistical analysis used Wilcoxon's ranking test for the non parametric, unpaired

data (Mann-Whitney test). Differences were considered significant at *P* values of <0.05.

5.12.4 RESULTS

All patients showed villous atrophy on histological examination of their jejunal and duodenal mucosal biopsies and they were all negative for Giardia. The disaccharidase activities were reduced in all patients (Table 5.12.2).

A plateau level of 5 ± 0.27 APE of tracer enrichments was achieved within one h of infusion for both IV and IG routes and remained so throughout the infusion period until biopsies were obtained at endoscopy (Figure 5.12.1).

Distal duodenal mucosal protein synthesis rate was markedly elevated in coeliac patients compared with control subjects whether determined by incorporation of the IV or IG infused tracers. IV tracer, coeliac vs. control 3.58 ± 0.45 vs. 2.26 ± 0.22 %/h $P<0.05$; IG tracer 6.25 ± 0.52 vs. 2.34 ± 0.52 %/h, $P<0.01$ (Figure 5.12.2). Labelling of mucosal intracellular amino acids was higher when tracer was given IG than IV but there were no differences between the control subjects and coeliac patients (Figure 5.12.3), suggesting that the higher rate of protein synthesis measured with IG tracer in the coeliac patients was not the result of differential precursor labelling or luminal tracer malabsorption.

TABLE 5.12.2 Disaccharidase activities in jejunal biopsies from the coeliac patients studied.

Patient	Lactase	Sucrase	Maltase
Normal range	1-12	3-15	10-55
1	0.1	0.5	2
2	1.6	4.9	1.9
3	0.2	0.3	2
4	0.1	0.2	1
5	0.2	0.9	5
6	0.0	1	4
7	0.0	0.4	2
8	0.3	2.9	1.0

The disaccharidase activities are expressed as international unit/gm of wet weight (IU/gm).

Protein/DNA ratios were reduced in the patients with coeliac disease compared to the control subjects, 9.2 ± 1.6 mg/ug vs. 13.0 ± 2.2 mg/ug, respectively $P < 0.05$ (Figure 5.12.4). The protein synthetic capacity, RNA/protein ratio, was elevated in the coeliac patients compared to the control subjects, 172 ± 7.5 ug/mg vs. 137.1 ± 5.7 ug/mg respectively, $P < 0.005$ (Figure 5.12.5).

Whole-body protein breakdown in coeliac disease was 206 ± 16 umol leucine/kg/h and in the control subjects 196 ± 44 umol leucin/kg/h, the difference between the 2 groups not being statistically significant, suggesting that the effect of coeliac disease is localized to the intestinal mucosa.

The keto acid/amino acid ratios after IG delivery of tracer amino acids were significantly lower in coeliac patients for both tracers; KIC/leucine and KIV/valine were 0.95 ± 0.03 and 0.94 ± 0.03 in controls and 0.74 ± 0.02 and 0.79 ± 0.06 in coeliac patients respectively $P < 0.05$ (Figure 5.12.6).

Duodenal mucosal tissue in coeliac patient showed a high concentration of RNA relative either to DNA or to protein, indicative of a substantial capacity for protein synthesis. The rates of protein synthesis in terms of RNA were 0.33 for the IV route and 0.58 mg protein.h^{-1}.mg RNA^{-1} (Table 5.12.3).

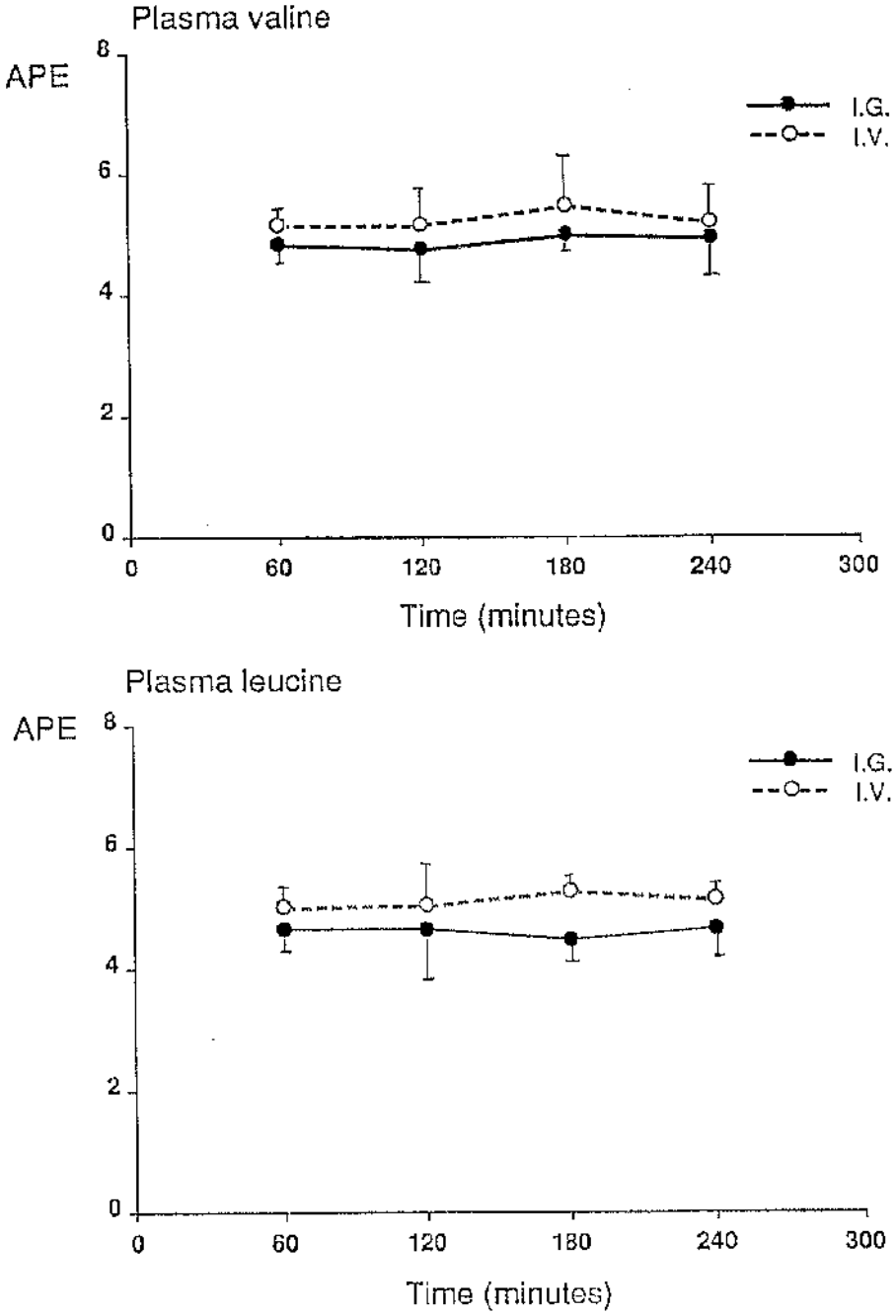


Figure 5.12.1 Plasma labelling of valine and leucine tracers in coeliac patients.

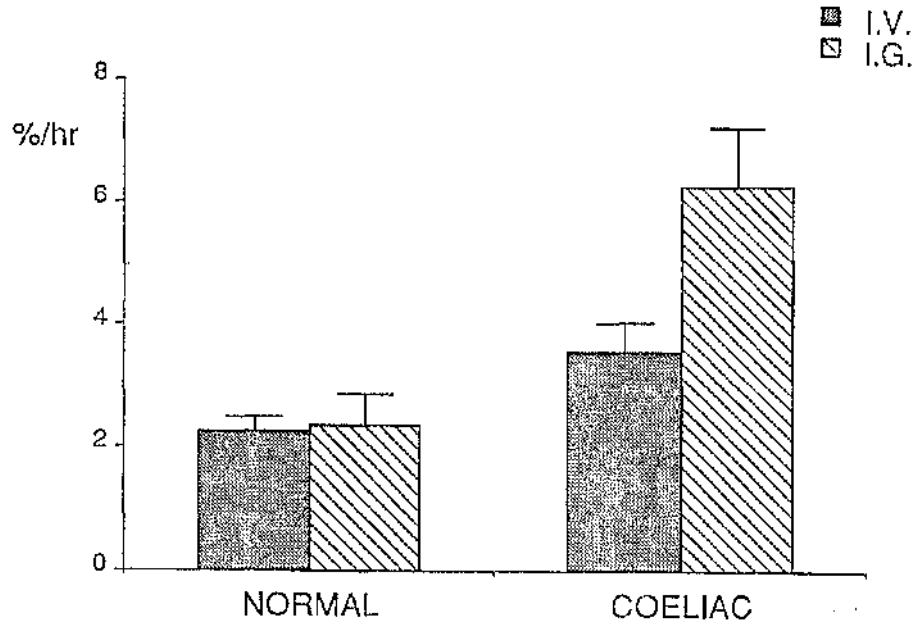


Figure 5.12.2 The rates of intestinal mucosal protein synthesis in controls and coeliac patients.

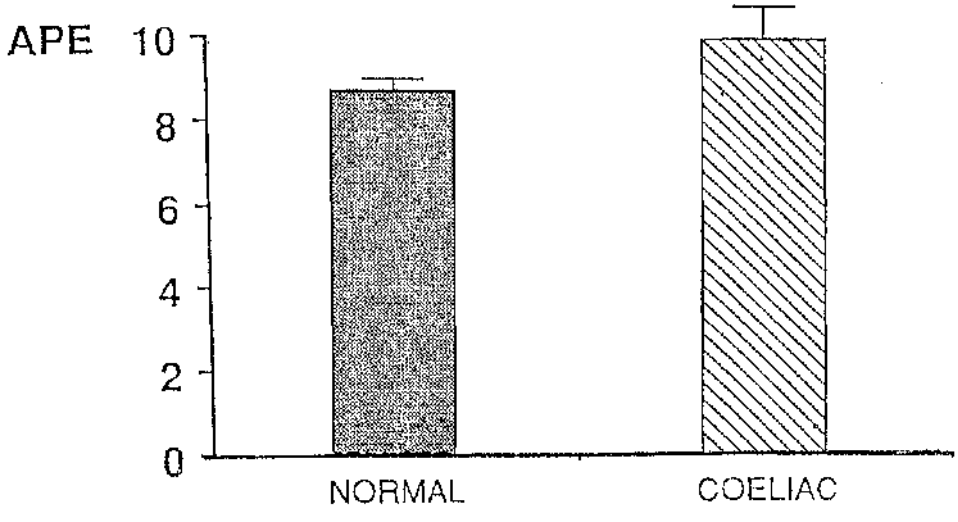


Figure 5.12.3 Intestinal mucosal free tracer enrichment during intragastric delivery of tracers in controls and coeliac patients.

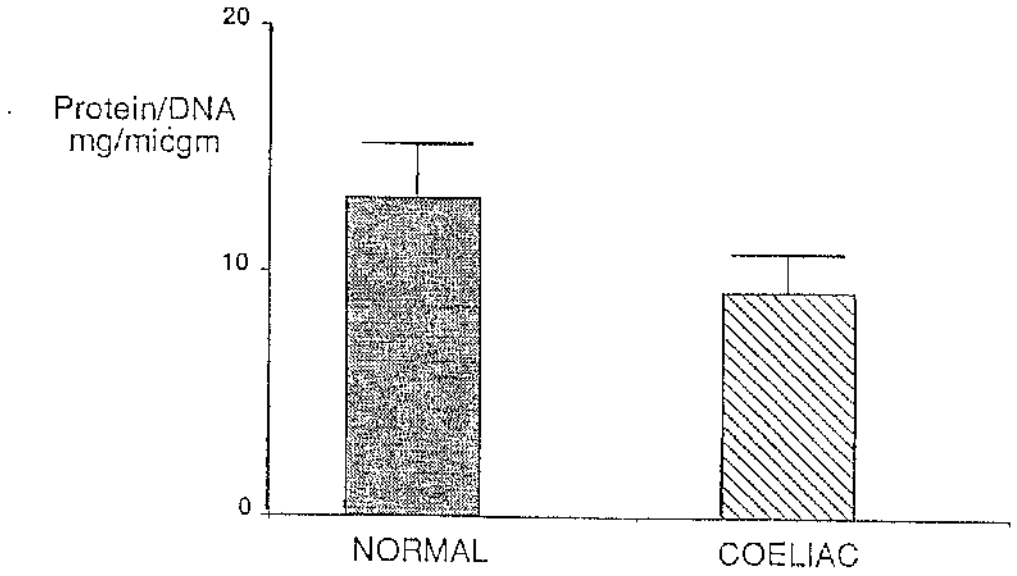


Figure 5.12.4 Duodenal mucosal cellular size in controls and coeliac patients.

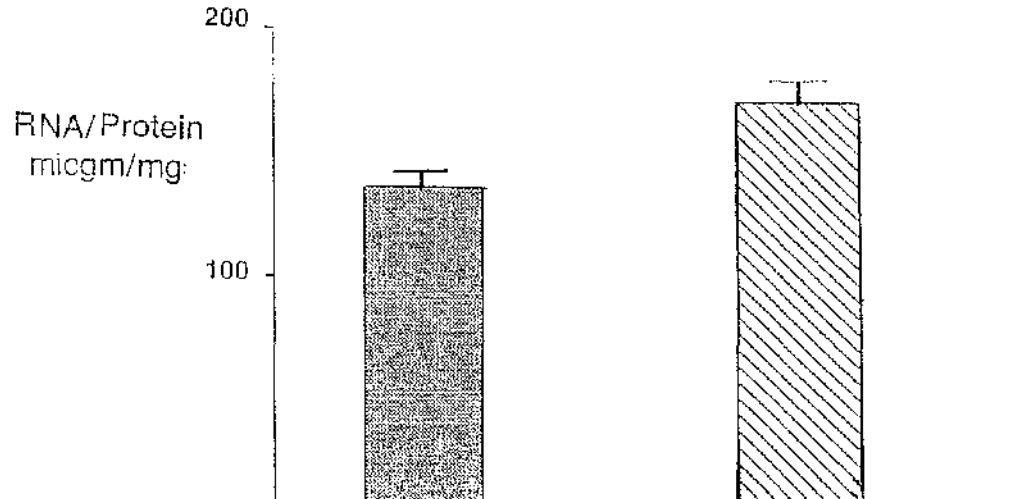


Figure 5.12.5 Protein synthetic capacity in controls and coeliac patients.

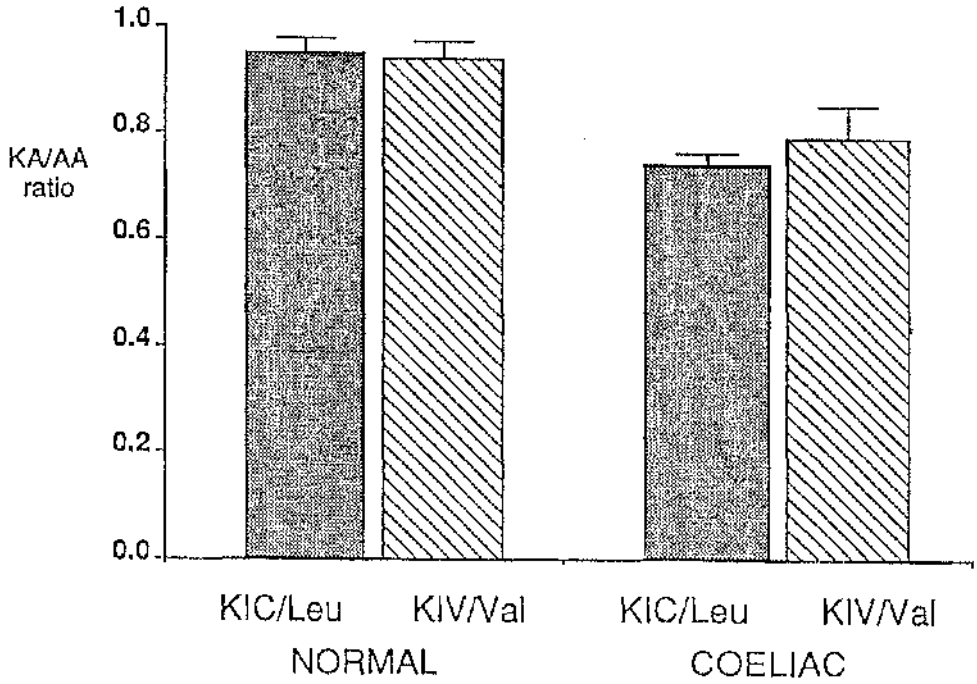


Figure 5.12.6 Keto acid / amino acid labelling ratios after intragastric delivery of tracer amino acids in controls and coeliac patients.

TABLE 5.12.3 Mucosal tissue composition and protein synthetic capacity in coeliac patients.

Protein $mg.g^{-1}$	DNA $mg.g^{-1}$	RNA $mg.g^{-1}$	Prot. synt. capacity mg $prot.h^{-1}.mg\ RNA^{-1}$
12.67 ± 0.15	0.85 ± 0.06	1.35 ± 0.06	0.33 ± 0.05 IV
			0.58 ± 0.06 IG

5.12.5 DISCUSSION

This study presents for the first time the effect of untreated coeliac disease on the rate of small bowel mucosal protein synthesis *in vivo*. The rate of protein synthesis by the small intestinal mucosal cells was higher in coeliac patients in spite of the smaller size of the enterocytes. The increased rate of mucosal protein synthesis in coeliac patients is in agreement with the results of an *in vitro* study by Jones et al.(209). They cultured jejunal mucosa from patients with coeliac disease, treated coeliac disease and controls with ^{14}C-labelled leucine for 24 h and found that protein synthesis by mucosa from untreated coeliac patients was significantly greater than by control mucosa.

We calculated the mucosal protein synthesis as fractional rate relative to the intracellular tracer enrichment. Ideally the precursor should be the aminoacyl tRNA which is difficult to measure (178,179). Therefore, we used free mucosal

intracellular tracer enrichment as a surrogate for tRNA (Chapter 10).

Presentation of the tracer amino acid to the apical (luminal) side of the enterocytes seems to be associated with higher protein synthetic rate than on presentation of these tracers on the basolateral (intravascular) side. This finding was unexpected and is not seen in normal healthy subjects, probably due to the preferential channelling of amino acids from the luminal side because of the immaturity of the enterocytes in coeliac disease.

Coeliac disease is caused by damage to the villous epithelium of the small intestinal mucosa, giving a characteristic histological appearance (210) in response to the ingestion of dietary gluten which is present in most cereal grains (211). The condition may be accompanied by malabsorption of many nutrients, but the clinical picture only appears when the small intestinal damage is severe or deficiencies appear (12). There is evidence of increased mucosal cell turnover with marked proliferation of normal crypt cells (212), and our findings are in agreement with this. So far the metabolic capacity of these cells remains unclear.

Our diagnostic criteria for the diagnosis of coeliac disease depended on jejunal biopsy rather than endoscopic distal duodenal biopsy. Endoscopic biopsies tend to be smaller in size, difficult to orientate and interpret (210,213,214,215). They are also of lower disaccharidase activities comparing to the jejunal biopsies, particularly so

for the lactase enzyme (216,217). The values of these disaccharidase activities are shown in table (5.12.4) **(218).**

TABLE 5.12.4 Normal values of disaccharidase activities in the duodenal and jejunal mucosa.

Site	Lactase	Sucrase	Maltase
Duodenum	0.5-7.4	1.3-10.7	5.8-35
Jejunum	1-12	3-15	10-55

The KIC/Leu and KIV/Val ratios were below 1 and were significantly lower in coeliac patients suggesting more dilution of the keto acids resulting from elevated rate of protein breakdown and turnover. The intestinal mucosa showed evidence of atrophy as manifested by the decreased protein/DNA ratio. The extrapolation that the protein/DNA ratio reflects epithelial cellular size has been confirmed by conducting histomorphometric studies on the small intestinal mucosal biopsies from the coeliac patients and the control subjects (Chapter 13). The capacity of protein synthesis (RNA/protein) is elevated in the coeliac patients. This is probably a reflection of the immaturity of the enterocytes lining the villus and/or elevated rate of mucosal protein breakdown. In conclusion the small intestinal mucosal cells in coeliac disease seem to be immature, hence there is poor development of absorptive and metabolic functions.

The results of this study provides further insight into the changes in protein metabolism at tissue level which occur

in patients with coeliac disease. The tissue biopsies in these studies are made up, not only of intestinal epithelial cells but also comprise many other cell types e.g mesenchymal cells, immunocytes and fibrous tissue. The contribution of the lamina propria to the protein synthesis is negligible, as the rate of mucosal protein synthesis was the same in all the untreated coeliac patients in spite of the varying degree of inflammatory cellular infiltration in those patients. Furthermore, it has been found by analytical subcellular fractionation that most of the net protein synthesis takes place in the enterocytes and only negligible amounts synthesised by the cells of the lamina propria **(141)** Further developments of the current technique of measuring mucosal protein synthesis *in vivo* is required to allow the contribution of each cell type to the rate of protein synthesis in the whole biopsy to be determined.

Jones et al. found that protein synthesis by treated non-responsive coeliac mucosa was significantly less than untreated coeliac mucosa but greater than control mucosa **(209)**. Further *in vivo* study is required to reevaluate the rate of protein synthesis in patients with coeliac disease who have responded clinically and histologically to a gluten free diet as well as in those patients who have not responded to this diet.

5.13.6 SUMMARY

We have applied a robust, reproducible method for the measurement of protein synthesis in the gastrointestinal

mucosa to investigate possible differences between the rate of duodenal mucosal protein synthesis in coeliac patients and normal control subjects. Eight patients, means ± SD (51 ± 10 y, 57 ± 11 kg, 160 ± 6 cm) with newly-diagnosed untreated coeliac disease and seven control subjects (48 ± 11 y, 71.5 ± 12 kg, 172 ± 10 cm) received primed, continuous IG and IV infusions of L-[1-^{13}C]leucine and L-[1-^{13}C]valine after an overnight fast. Distal duodenal biopsies were obtained at endoscopy performed after 240 min of infusion. Protein synthesis was calculated from protein labelling relative to intracellular free amino acid enrichment, after appropriate mass spectrometric measurements. Rates of duodenal protein synthesis were significantly greater in coeliac patients than in control subjects (IV tracer, coeliac vs. control, 3.58 ± 0.45 vs. 2.26 ± 0.22 $\%h^{-1}$, $P < 0.05$; IG tracer, 6.25 ± 0.97 vs. 2.34 ± 0.52 $\%h^{-1}$ respectively, $P < 0.01$). The rates of mucosal protein synthesis calculated on the basis of the tracer infused via the IG route were higher in patients with coeliac disease than in control subjects. Tissue protein/DNA ratios were significantly reduced in coeliac patients (coeliac vs. control, 9.2 ± 1.6 mg/ug vs. 13.0 ± 2.2 mg/ug respectively, $P < 0.05$) suggesting smaller mucosal cell size in coeliac patients. Despite the villous atrophy and reduced cell size observed in coeliac disease, the rates of mucosal protein synthesis are markedly elevated. These results suggest that a high rate of protein synthesis may be adaptive to a high rate of protein breakdown or mucosal cell loss in coeliac patients.

CHAPTER 13

CRYPT CELL HISTOMORPHOMETRY IN COELIAC DISEASE: COMPARISON WITH MUCOSAL PROTEIN AND DNA COMPOSITION AND CORRELATION TO VITAMIN B12 AND FOLATE STATUS

5.13.1 INTRODUCTION

Coeliac disease is characterized by malabsorption and typical but non-specific histological changes in the small intestinal mucosa in response to ingestion of dietary gluten. The etiology of coeliac disease remains unresolved but it seems that genetic and immunologic factors play an important role in the pathogenesis of this disease (211).

The lesions are always most severe in the upper part of the small intestine and become progressively less marked distally (212). The pathological changes are characterized by sub-total villous atrophy, abnormal surface mucosal cells, increased cellularity of the lamina propria; mainly lymphocytes and plasma cells, and elongation of the crypts of Lieberkuhn (205,207). Under light microscopy the crypts cell damage is recognized by reduction in cell height, often associated with increased cytoplasmic basophilia, vacuolation and nuclear irregularity with loss of polarity. There is pronounced mitotic activity with a substantially increased mitotic index (212,219).

The most important diagnostic feature of coeliac disease is that a measurable improvement in villous architecture

takes place when gluten is withdrawn from the diet (211). Direct visual observation of histological sections under light microscopy lacks discrimination compared to histometry (220). Therefore a more sensitive measure of the architectural changes in coeliac disease would be invaluable to follow up response to treatment.

The small intestinal mucosa is a tissue with the most rapid turnover of any in the body (205) and as such it resembles the haemopoetic system. There is an analogy between the intestinal mucosa and the haemopoetic system and a nomenclature was proposed (221) based on haematological principles. The crypt cells in the germinative zone can be called "enteroblast" and the adult absorptive cells, which show no mitosis and have limited life span resembling peripheral blood cells, then be termed "enterocytes". The whole process of cell turnover can be termed "enteropoeiesis" (222).

Lack of adequate levels of vitamin B_{12} or folate is the cause of megaloblastic anaemia secondary to vitamin deficiency (223). Structural or functional damage in the upper third of the small intestine, such as in coeliac disease, is associated with folate deficiency (224). Both vitamin B_{12} and folic acid are required for synthesis of thymidylate and therefore, of DNA (224). Morphological changes in the "enterocytes" similar to those seen in the erythrocytes would be expected in response to vitamin deficiency.

5.13.2 AIMS

The aims of this study were; 1) to asses the nuclear, cellular and cytoplasmic surface area of crypt cells in the small intestinal mucosa in coeliac disease patients and controls; 2) to compare these characteristics with the protein and DNA composition of the enterocytes; 3) to asses the effect of gluten withdrawal from the diet on the histomorphometric characteristics of crypt cells in the small intestinal mucosa in coeliac disease patients; and 4) to correlate these histomorphometric measurements to the B_{12} and folate status and mean corpuscular volume (MCV) in the control subjects and patients studied.

5.13.3 PATIENTS AND METHODS

Small intestinal mucosal samples were obtained from 40 subjects, 21 untreated coeliac disease, means ± SD (42 ± 14 y, 66.5 ± 12 kg, 169 ± 11 cm, male/female 10/9) and 19 controls (48 ± 10 y, 61 ± 11 kg, 161 ± 6.5 cm, male/female 10/11).

DNA and protein composition of the intestinal mucosa were determined in 7 control subjects (Table 4.10.1) and 8 coeliac patients (Table 5.12.1) as previously described (Chapter 12).

Small intestinal biopsies from the jejunum were taken by Crosby Kugler capsule and from the distal duodenum via the endoscope and immediately fixed in Buin's solution (saturated aqueous picric acid 75 ml, 40% formaldehyde 25 ml and glacial acetic acid 5 ml) (225) after mounting them on filter paper,

villous side upwards. The biopsies were carefully oriented and embedded in paraffin wax and sections (4u) were cut on the same microtome and the speed of cutting kept steady. These sections were stained with haematoxylin and eosin according to standard procedures (226) for routine histopathological examination. All sections were viewed with a Leitz microscope type 307-148.001 (Leitz-Wetzler, Germany) through a X500 objective; appropriate photomicrographs were taken with an Orthomat camera (Leitz-Wetzler, Germany) and recorded on Kodak Tec-Pan 35 mm film. With this level of magnification, which was used throughout, a measurement of 1mm on the microphotograph was equivalent to 4.4 u on the section.

The nuclear and cellular surface area were measured in 50 crypt cells in each section. The cytoplasmic surface area was obtained by subtracting the nuclear surface area from the cellular surface area.

In the sections studied the nuclear shape was elliptical therefore the surface area was calculated from the expression;

$$A = \pi ab = 3.14159\ ab = 3.142\ ab,$$

where A is the area, a the long radius and b the short radius (227).

The surface area of the crypt cells was measured by the tracing method (220). The crypt cells were grouped into sectors of 5 adjacent cells and drawings were made on acetate sheets and the shapes cut out neatly with scissors and weighed. On the same acetate sheet ten 10 x 10 mm squares

were drawn, cut out and weighed using a sensitive weighing balance (Unimatic single pan weighing balance, Stanton Instruments Ltd, London). The weight of these squares was 176 ± 4 ug and the surface area 1936 u^2 (10 mm x 10 mm = 44 u x 44 u = 1936). The crypt cell's sectors were weighed using the same balance and the surface area was calculated from the expression;

Sector's surface area = Sector's Weight x 1936/176

and divided by 5 to obtain the cellular surface area.

The measurement of nuclear surface area of the crypt cell was repeated in 6 patients 12-18 months after the introduction of gluten free diet to assess the effect of gluten withdrawal on the histomorphometry.

Blood samples were obtained at the time of biopsy from 12 normal subjects (age 38 ± 14 y, weight 62.5 ± 10.5 kg, height 166.5 ± 10 cm and male/female ratio 4/8) and 13 coeliac disease patients (age 45.5 ± 10 y, weight 62 ± 10 kg, height 162 ± 7 cm and male/female ratio 6/7) and sent for analysis. Vitamin B_{12}, serum folate and red blood cell (RBC) folate were measured by a radioassay method (Becton Dickinson SimulTRAC Radioassay kit, New York 10962-1294) (228,229) and the MCV was measured according to the standard procedure using automated counter in the Haematology laboratory.

Results are expressed as ranges and/or means ± SD. Comparison of the cellular, cytoplasmic and nuclear surface area as well as protein/DNA, cytoplasmic/nuclear surface area ratios and serum folate levels between control and coeliac disease was achieved by using the non-parametric paired test

(Mann-Whitney U test). The changes pre and post therapy were carried out using paired Student t test. Correlation of the nuclear surface area to the B_{12}, serum folate, RBC folate and MCV was carried out using the non-parametric Spearman's rank correlation test and the Two-tailed t test for *P* values. Difference between values were considered to be statistically significant at a *P* value of < 0.05.

5.13.4 RESULTS

The nuclear surface area in the coeliac disease patients was higher than in the control subjects (coeliac disease vs. controls; $125.52 \pm 21.78\ u^2$ vs. $44.47 \pm 10.75\ u^2$, $P < 0.008$). There was no significant difference in the cellular surface area between the two groups (coeliac disease vs. controls ; $602.06 \pm 101.09\ u^2$ vs. $556.65 \pm 12.52\ u^2$, *P*=NS) (Figure 5.13.1).

Protein/DNA ratio was reduced in the coeliac disease patients compared with the controls (coeliac disease vs. controls; 9.2 ± 1.6 mg/ug vs. 13.0 ± 2.2 mg/ug respectively, $P < 0.05$) (Figure 5.12.4). The cytoplasmic surface area/nuclear surface area ratio was again reduced in the coeliac disease patients compared with the controls (coeliac disease patients vs. controls; $3.67 \pm 0.58\ u^2/u^2$ vs. $9.11 \pm 0.84\ u^2/u^2$, $P < 0.008$) suggesting that mucosal cell size is reduced in coeliac disease patients (Figure 5.13.2).

Table 5.13.1 shows the levels of vitamin B_{12}, serum folate and RBC folate as well as the MCV. Serum folate level

was low in coeliac disease (coeliac disease patients vs. controls; 2.92 ± 0.83 vs. 5.48 ± 2.1 ng/ml, P <0.03).

TABLE 5.13.1 Vitamin B12, serum folate and RBC folate levels and MCV values of coeliac patients and controls.

	NORMAL	COELIAC	CONTROL
VITAMIN B_{12}	150-730	119-693	217-625
(pg/ml)	440	376.38	404.82
	± 290	± 181.97	± 135.35
SERUM FOLATE	2.2-11.4	1.8-3.7	2.2-9.9
(ng/ml)	6.8	2.92*	5.48
	± 4.6	± 0.83	± 2.1
RBC FOLATE	106-614	69-426	120-583
(ng/ml)	360	224.64	368.42
	± 254	± 117.13	± 145.3
MCV	76-96	76.8-96.8	81.2-94.5
(fl)	86	88.48	89.28
	± 10	± 5.78	± 3.85

* Coeliac disease vs. control; P < 0.03

levels are expressed as ranges and means ± SD

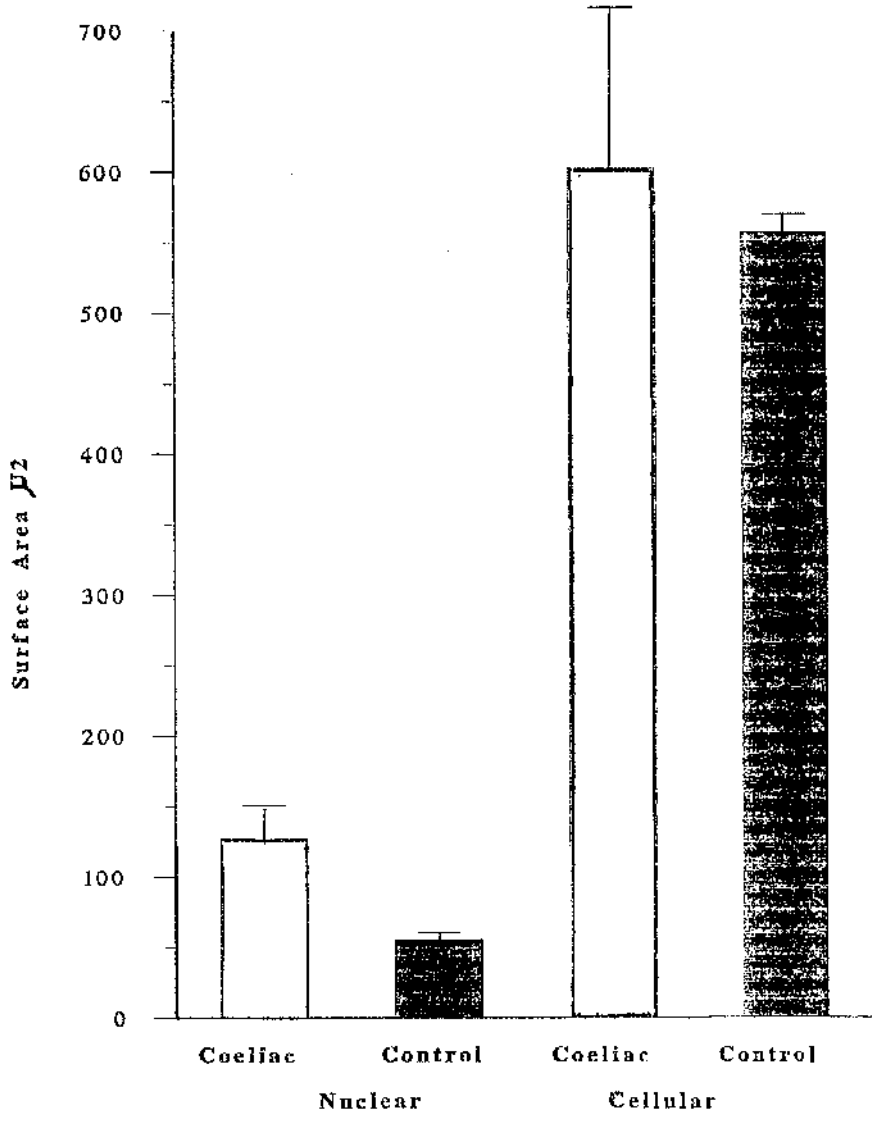


Figure 5.13.1 Nuclear surface area and cellular surface area of the crypt cells in coeliac patients and control subjects.

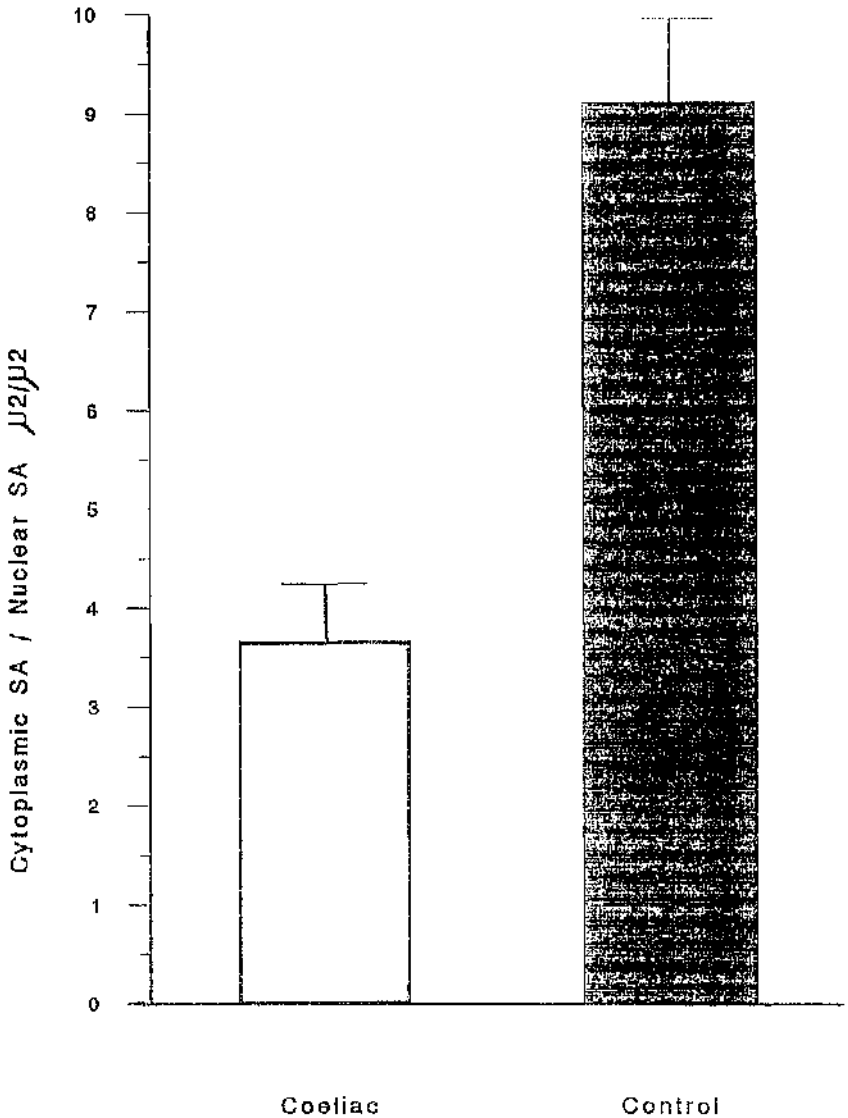


Figure 5.13.2 Cytoplasmic surface area/nuclear surface area ratio of the crypt cells in coeliac patients and normal controls.

The correlation between nuclear surface area and vitamin B_{12}, serum folate, RBC folate and MCV in coeliac disease patients and controls are shown in figures 5.13.3, 5.13.4, 5.13.5 and 5.13.6 respectively. The serum folate levels in coeliac disease showed a significant negative correlation with the nuclear surface area ($y=6.08-0.03x$, $r^2=0.62$, $P<0.02$)

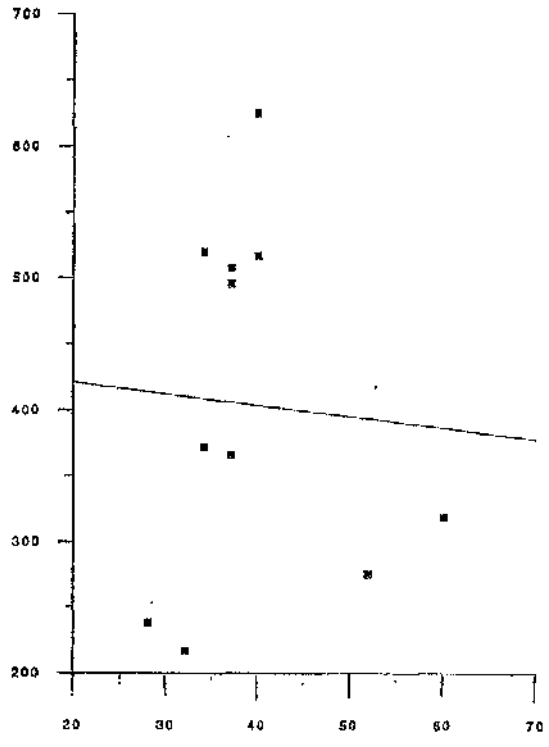
The introduction of gluten free diet resulted in clinical response in the patients who were compliant with the treatment. However, the histological response as assessed by routine light microscopy was variable. The nuclear surface area of the crypt cells showed a reduction from 125.97 ± 12.34 u^2 down to 72.05 ± 4 u^2, $P < 0.001$ (Figure 5.13.7).

Control

y=438.75-0.87x

r^2=0.003

p=NS



Vitamin B_{12}

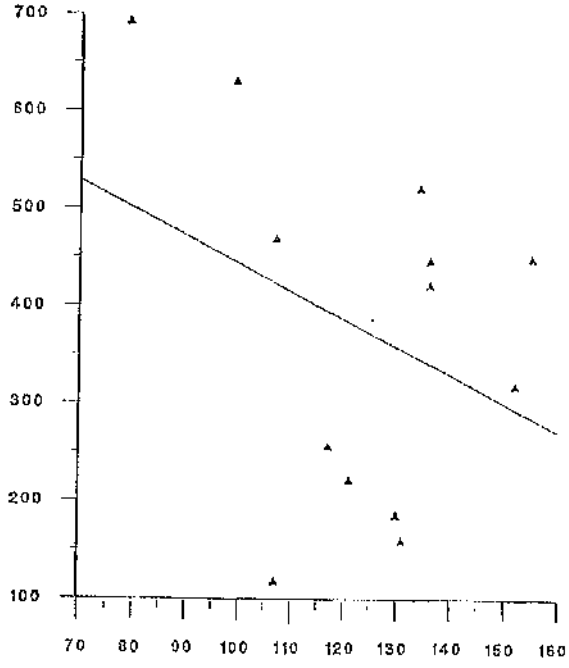
pg/ml

Coeliac

y=728.85-2.86x

r^2=0.11

p=NS



Nuclear surface area u^2

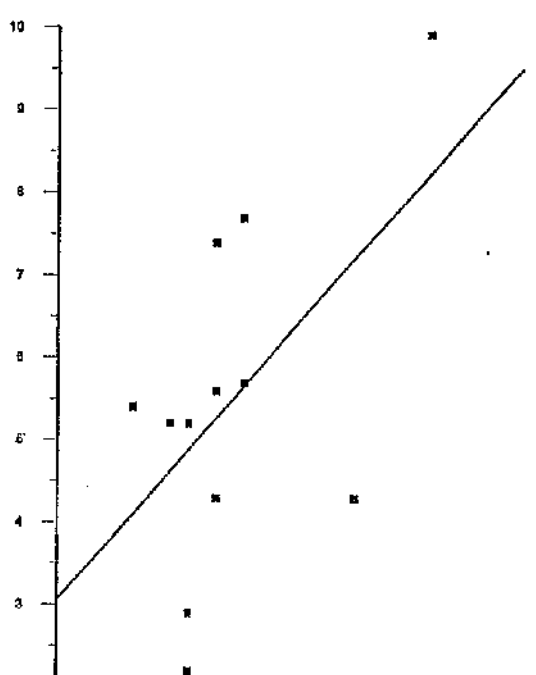
Figure 5.13.3 Correlation between the serum vitamin B_{12} level and crypt cell nuclear surface area in control subjects and coeliac patients.

Control

$y=0.50+0.13x$

$r^2=0.30$

$p=NS$



Serum folate

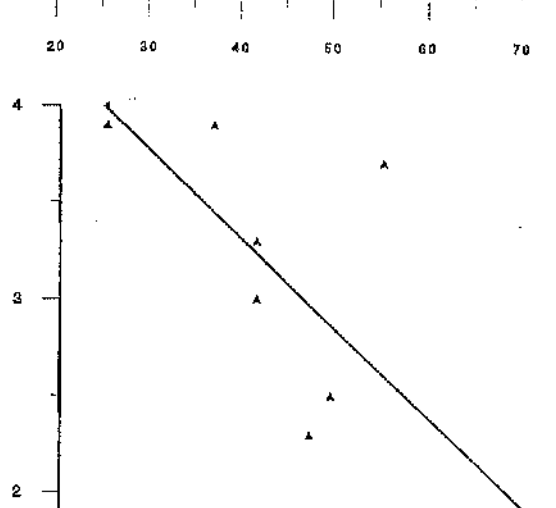
ng/ml

Coeliac

$y=6.08-0.03x$

$r^2=0.62$

$p<0.02$



Nuclear surface area u^2

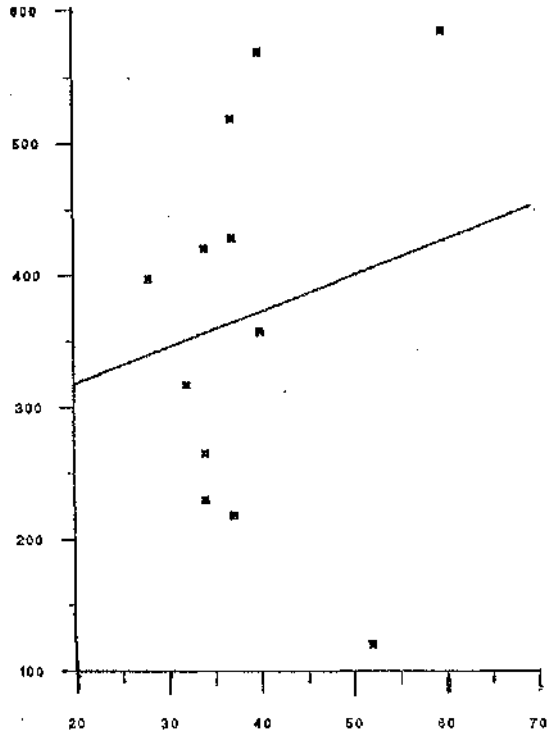
Figure 5.13.4 Correlation between the serum folate level and crypt cell nuclear surface area in control subjects and coeliac patients.

Control

y=263.79+2.70x

r^2=0.03

p=NS



RBC folate

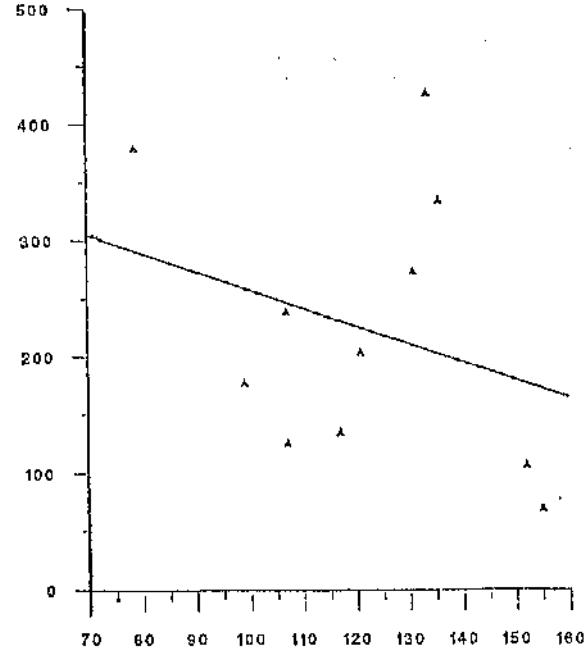
ng/ml

Coeliac

y=413.18-1.55x

r^2=0.09

p=NS



Nuclear surface area u^2

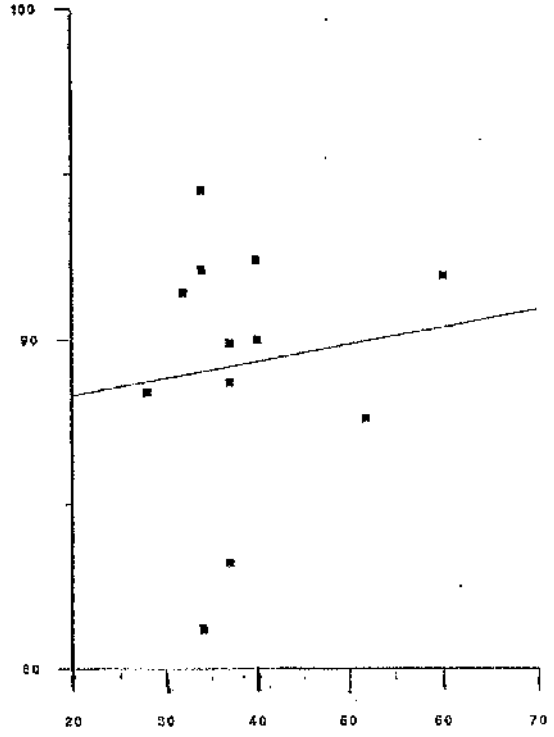
Figure 5.13.5 Correlation between the red blood cell folate level and crypt cell nuclear surface area in control subjects and coeliac patients.

Control

$y=87.31+0.05x$

$r^2=0.01$

p=NS



MCV

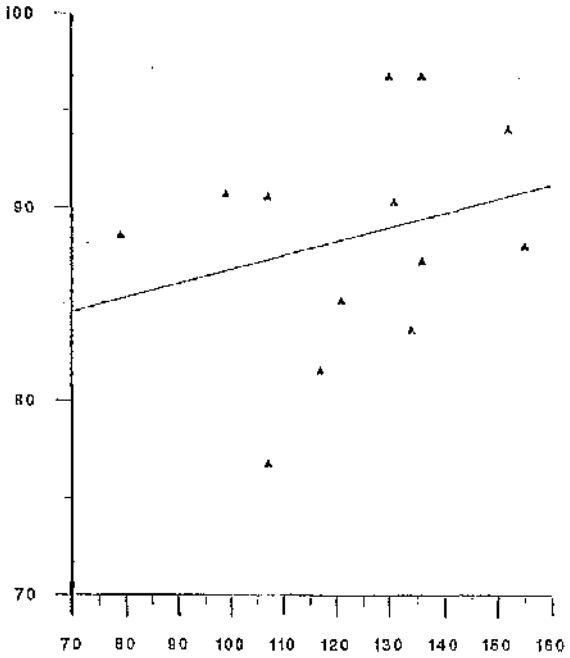
fl

Coeliac

$y=79.54+0.07x$

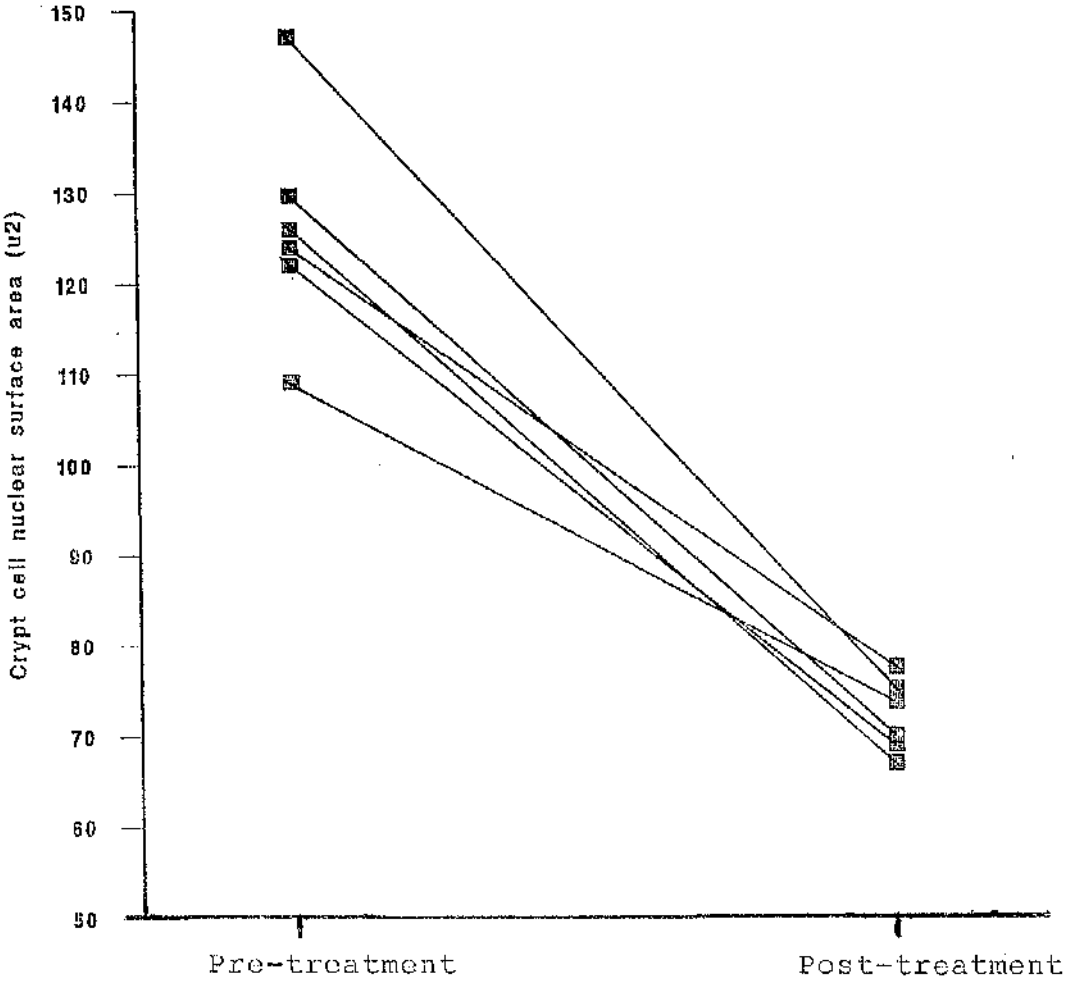
$r^2=0.07$

p=NS



Nuclear surface area u^2

Figure 5.13.6 Correlation between the MCV and the crypt cell nuclear surface area in control subjects and coeliac patients.



$P < 0.001$

Figure 5.13.7 The effect of gluten-free diet on the nuclear surface area of the crypt cell in patients with coeliac disease.

5.13.5 DISCUSSION

Histometry, the measurement of tissue, is particularly suitable for certain histological investigations. It offers objectivity and makes statistical analysis easier as direct visual observation lacks discrimination and is inconsistent compared to histometry. It also facilitates the comparison of histological studies from different centres (220).

Intestinal biopsy is of central importance in the investigation of coeliac disease. Most studies concentrated on the histological abnormality of the villous architecture in the form of villous atrophy associated with crypts hyperplasia and increased prominence of the intraepithelial lymphocytes and other leukocytes (230). Information on the histomorphometric characteristics of the crypts cells in coeliac disease is lacking apart from the mention of high mitotic index (212,231).

The DNA in the nucleus is packaged into 46 chromosomes in the nucleated diploid human cells and it is the same for all cells that have a diploid set of chromosomes (36). This study showed significant increase in the nuclear surface area in the coeliac disease patients which is probably a reflection of increased mitotic activity and so the chromosomes are not densely packaged as in the resting cells.

The change in the nuclear surface area of the crypt cell following the introduction of gluten free diet provides an early indicator of the response to this therapy in coeliac disease.

The reduced cytoplasmic surface area and protein content of the small intestinal mucosal cells is an indication of the immaturity of the mucosal cells in coeliac disease and could be more sensitive than direct light microscopy.

In our control group the levels of vitamin B_{12}, serum folate and RBC folate were within the normal ranges therefore no significant correlation with the MCV.

In coeliac disease the pathological changes tend to affect the proximal part of the small intestinal mucosa more than the distal part and the terminal ileum, and as such the absorptive capacity of the proximal part of the small intestine is affected to a greater extent. As the folate and iron are absorbed from the proximal part of the small intestine and vitamin B_{12} is absorbed from the terminal ileum, therefore in coeliac disease there is deficiency of folate and iron rather than vitamin B_{12} unless the disease is so sever and extensive to involve the terminal ileum. About one third of coeliac disease patients have a low cobalamine level (232). In our coeliac disease patients the cobalamine levels were lower than the controls, but the difference was not significant (Table 5.13.1). Because of the combination of iron and folate deficiencies the MCV values in the coeliac disease patients were within the normal range as a result of the dimorphic picture. Furthermore, splenic atrophy in coeliac disease is common and may result in changes in the blood film like target cells, Howel-Jolly bodies, RBC fragments and erythroblasts which affect the MCV (232). It seems that the nuclear surface area of the crypt cell in

coeliac disease is affected by the folate status and not the ferrous status.

Further studies are needed to measure the small intestinal mucosal tissue folate and iron levels and correlate these levels directly with the histomorphometric characteristics of the crypt cells in coeliac patients.

5.13.6 SUMMARY

We performed histomorphometry on the crypt cells of small intestinal mucosal samples obtained from patients with untreated coeliac disease and normal subjects. The histomorphometric characteristics were; compared with the protein and DNA composition of the intestinal mucosa; correlated to the vitamin B_{12} and folate status; and repeated after the introduction of gluten free diet. Jejunal and distal duodenal biopsy specimens were obtained from 40 subjects (19 controls and 21 patients with untreated coeliac disease) and analysed morphometrically by light microscopy using the tracing method. DNA and protein composition of the intestinal mucosa were assessed in a group of 7 controls and 8 coeliac disease, and vitamin B_{12} and serum folate as well as red cell folate were measured in the remaining subjects (13 coeliac disease and 12 controls). Histomorphometry was repeated in 6 patients with coeliac disease after the introduction of gluten free diet. The nuclear surface area in coeliac disease was significantly greater than in the controls (coeliac disease vs. controls, means ± SD, 125.52 ± 21.78 u^2 vs. 44.47 ± 10.75 u^2, $P < 0.008$). There was no

significant difference in the cellular surface area between the two groups. Protein/DNA ratio and cytoplasmic/nuclear surface area ratio were both reduced in patients with coeliac disease compared to controls (9.2 ± 1.6 mg/ug vs. 13.0 ± 2.2 mg/ug, $P < 0.05$; 3.67 ± 0.58 u^2/u^2 vs. 9.11 ± 0.84 u^2/u^2, $P<0.008$ respectively). Serum folate was low in coeliac disease patients (coeliac disease vs. controls 2.92 ± 0.83 vs. 5.48 ± 2.1, $P < 0.03$) and showed significant negative correlation with the nuclear surface area ($y=6.08-0.03x$, $r^2=0.62$, $P < 0.02$). No significant correlation was found between the nuclear surface area and vitamin B_{12}, serum folate and red blood cell folate in the normal subjects nor there was significant correlation between nuclear surface area in the coeliac disease patients and vitamin B_{12} and red cell folate. Nuclear surface area was reduced after the introduction of gluten free diet (pre-treatment vs. post-treatment; 125.97 ± 12.34 u^2 vs. 72.05 ± 4 u^2, $P < 0.001$). These results suggest that the nuclear surface area in coeliac disease is greater than normal, probably a reflection of increased mitotic activity. Serum folate was low in coeliac disease and correlated negatively well with the nuclear surface area of the crypt cells. The reduction in the nuclear surface area after withdrawing gluten from the diet can be used as an early indicator of response to this therapy.

SECTION VI

APPLICATION OF THE TECHNIQUE OF SMALL INTESTINAL MUCOSAL PROTEIN SYNTHESIS IN ALCOHOLIC LIVER DISEASE PATIENTS

CHAPTER 14

SMALL INTESTINAL MUCOSAL PROTEIN SYNTHESIS IS DEPRESSED IN PATIENTS WITH ALCOHOLIC LIVER DISEASE AND CORRELATES WITH WHOLE-BODY PROTEIN BREAKDOWN

6.14.1 INTRODUCTION

Malnutrition is commonly associated with alcoholism (233,234). By the time patients have developed liver disease which is clinically obvious they often show evidence of protein-energy malnutrition (235,236) and other nutritional deficiencies (237). There is some difficulty in accurately defining the nutritional status in chronic alcoholism (238). Among the methods used are measurement of nitrogen status and metabolism by such indices as the ratio of concentrations of branched-chain to aromatic amino acids in plasma and the rate of plasma amino acid turnover obtained using labelled leucine (233). There is evidence of wasting of lean body mass in alcoholic liver disease, especially of skeletal muscle, but little is known of the involvement of the gut in the wasting process. Studies in rats provide evidence of depressed small intestinal mucosal protein synthesis (239) and intestinal protein synthesis (240,241) in response to alcohol suggesting the existence of similar deficits in man. Furthermore, little is known about the functional correlates of possible gastrointestinal involvement in man in response to chronic ethanol ingestion.

6.14.2 AIMS

The aims of this study were; 1) to measure indices of mucosal cell size and protein synthetic capacity and the rate of small intestinal mucosal protein synthesis, 2) to investigate links between protein turnover in the gut and those in the whole-body in patients with alcoholic liver disease and in normal healthy subjects, and 3) to correlate the small intestinal mucosal protein synthetic rates and whole-body protein turnover rates with the severity of alcoholic liver disease and the amounts of alcohol consumed.

6.14.3 PATIENTS AND METHODS

Eight patients with alcoholic liver disease were studied at the Department of Gastroenterology, Glasgow Royal Infirmary (Table 6.14.1). Each subject gave written consent after a full explanation of the nature of the study. Approval was obtained from the Ethics Committee of Glasgow Royal Infirmary University NHS trust.

The results from these patients with alcoholic liver disease were compared with the results obtained from a group of eight normal healthy subjects who had been consuming less than 10 units of alcohol a week and had normal liver function tests (Table 6.14.2).

All patients had regularly consumed in excess of 50 units of alcohol weekly until the day of admission to the hospital. Reasons for emergency hospital admission were jaundice, abdominal pain, ascites, weight loss, exhaustion or vomiting (Table 6.14.3). Patients with infection, encephlaopathy,

gastrointestinal bleeding, uncorrected coagulation status or any previous history of hepatic failure were excluded from the study. All patients showed clinical, biochemical and ultrasonic evidence of parenchymal liver damage (Table 6.14.4). Liver biopsy in 2 patients confirmed the diagnosis of alcoholic liver disease and in one of them fell just short of a diagnosis of hepatic cirrhosis. Screen for autoimmune (antismooth muscle antibodies, antimitochondrial antibodies and anti nuclear factor) and viral factors (hepatitis A, B & C) were negative in all patients. None of the patients was taking drugs that could affect the liver, apart from alcohol. All patients were started on a daily diet restricting sodium (to 40 mmol) and water (to 1 liter) with normal protein intake (100 gm) on admission to hospital.

TABLE 6.14.1 Characteristics of patients with alcoholic liver disease studied.

Patient	Age-Y	Sex	Weight-kg	Height-cm	%Ideal BW
1	45	M	67	172	98.38
2	41	F	63	157	111.70
3	42	M	54	162	85.31
4	44	M	74	177	104.52
5	45	M	78	164	121.68
6	37	M	98	180	135.36
7	31	M	86	177	121.47
8	45	M	72	175	103.30

% ideal BW : % ideal body weight (198)

They were also started on spironolactone (Aldactone; Searle, Bucks, England) 100-200 mg daily to control their fluid retention.

TABLE 6.14.2 Characteristics of normal subjects studied for small intestinal mucosal protein synthesis.

Patient	Age-Y	Sex	Weight-kg	Height-cm	%Ideal BW
1	57	M	65	158	105.01
2	53	M	85	185	112.58
3	59	M	70	178	98.18
4	45	M	56	172	82.23
5	54	M	87	185	115.23
6	24	F	63	166	102.77
7	21	M	62	162	97.95
8	40	F	79	160	135.97

% ideal BW : % ideal body weight (198)

To gain an insight into the severity of alcoholic liver disease in the patients studied I used a biochemical scoring system which involves measuring AST, ALT, serum bilirubin, serum albumin, gamma GT and vitamin B_{12} level (Table 6.14.5). I used this scoring system to correlate the severity of alcoholic liver disease with the small intestinal mucosal protein synthetic rates and whole-body protein turnover rates. It has been found that alcoholic liver disease is characterised by distinct patterns of AST and ALT elevations (243,244), and that the serum vitamin B_{12} concentration is a

useful marker for the severity of necrosis observed in liver biopsy (245). Therefor I have included these indices in the scoring system.

TABLE 6.14.3 Alcohol consumption and reasons for hospital admission of patients with alcoholic liver disease.

Patient	Alcohol Consumption		Reason for Admission to Hospital
	Amount*	Duration+	
1	60	15	Jaundice & abdominal pain
2	56	18	Ascites, peripheral odema & exhaustion.
3	55	25	Ascites, Jaundice & weight loss
4	60	10	Ascites & Jaundice
5	50	15	Abdominal pain
6	60	16	Abdominal pain
7	80	8	Jaundice & withdrawal fits
8	75	30	Jaundice, vomiting, weight loss & ascites

*Amount : units per week

+Duration : in years

One unit of alcohol = 10 gm of ethanol,
30 ml of whisky,
100 ml of wine or
250 ml beer (242)

TABLE 6.14.4 Biochemical and clinical characteristics of patients with alcoholic liver disease.

Pt.	AST	ALT	Bili	GGT	Alb	Vit B_{12}	Ascites+	Hepar*
1	410	69	190	1070	32	1027	++	2
2	38	31	48	380	34	540	+++	4
3	158	80	15	2010	47	885	+++	1
4	94	28	818	335	34	738	+++	3
5	129	216	8	250	32	325	+	3
6	58	25	53	53	39	554	+	2
7	75	61	79	740	36	539	+	2
8	106	20	170	61	33	540	+++	4

* Hepatomegaly measured in cm below the costal margin

+ Degrees of ascites, + mild, ++ moderate and +++ severe

Normal Ranges:

AST	12-48 U/l
ALT	3-55 U/l
Serum bilirubin	3-22 umol/l
Serum albumin	35-55 g/l
GGT	<36 U/l
Vitamin B_{12}	150-730 pg/l

TABLE 6.14.5 Scoring system for the severity of alcoholic liver disease.

PARAMETER	SCORE			
	0	1	2	3
AST	normal	<2-fold	>2-fold	>3-fold
ALT	normal	<2-fold	>2-fold	>3-fold
Bilirubin	<35	>35	>70	>150
Albumin	>35	<35	<30	<25
GGT	normal	<2-fold	>2-fold	>3-fold
Vitamin B_{12}	<average	>average	>normal	>2-fold

0-6 mild

6-12 moderate

12-18 severe

L-[1-^{13}C]leucine 99 Atom % was dissolved in sterile, non-pyrogenic 0.9% NaCl solution (150 mmol/liter) immediately before administration and infused IV through a 0.20 u filter.

The subjects were studied in the morning after an overnight fast. A priming dose of L-[1-^{13}C]leucine 1mg/kg body weight was given IV over one min; a constant infusion of the same tracer at a rate of 1mg /kg body weight/h using 120 ml of 0.9% NaCl was then continued for 4 h. After 240 min of continuous infusion upper gastrointestinal endoscopy was performed and multiple distal duodenal biopsies were

obtained. The range of the pooled wet weight of biopsies was 56.1-88.0 mg. Further biopsies were taken for histology. Venous blood samples were taken at -15, 0, 60, 120, 180 and 240 min respectively. Venous blood samples were centrifuged and separated plasma kept in liquid nitrogen until analysis Figure 3.9.3 illustrates the experimental protocol followed in this study.

The labelling and concentrations of plasma phenylalanine, leucine and KIC were measured by standard GCMS techniques using t-BDMS derivatives (199). Mucosal tissue samples were frozen in liquid nitrogen immediately on sampling, and samples pooled for storage at $-70^{0}C$. The pooled mucosal tissue samples were analysed for concentration of protein, RNA, and DNA (190). The labelling with ^{13}C of leucine in hydrolysed protein was measured by using preparative GC to isolate the amino acid with IRMS of the CO_2 liberated by ninhydrin (185). Plasma protein from the pre-infusion samples was used to estimate basal body protein ^{13}C labelling for use in the calculation of mucosal protein synthetic rate.

Protein synthesis was calculated as a fractional rate and whole-body protein breakdown was calculated from plasma ^{13}C KIC as described previously (Chapter 9).

Values were expressed as means ± SD and mean (range). Statistical analysis used the non parametric, unpaired Student t-test, Spearman's rank correlation test or the two-tailed t test as appropriate. Differences were considered significant at P values of <0.05.

6.14.4 RESULTS

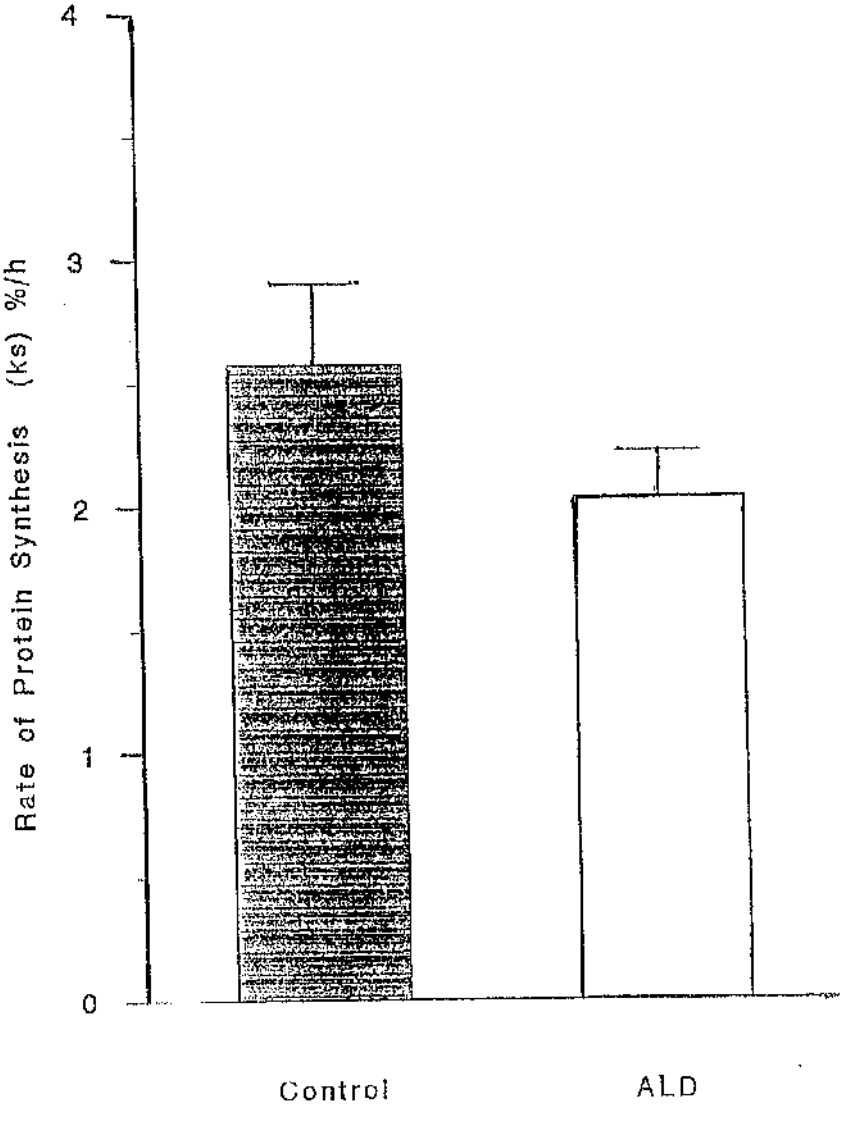
A plateau level of 5.1 ± 0.75 APE of L-[1-^{13}C]leucine enrichments was achieved within one h of infusion and remained so throughout the infusion period until biopsies were obtained at endoscopy.

Distal duodenal mucosal protein synthesis rate was found to be lower in the alcoholic liver disease patients than in the control subjects (alcoholic liver disease vs. control; 2.04 ± 0.18 vs. 2.58 ± 0.32 %/h, $P < 0.003$) (Figure 6.14.1).

The protein/DNA ratio, an index of cell size, was also lower in the patients with alcoholic liver disease than in the control subjects (9.23 ± 0.91 mg/ug vs. 13.0 ± 2.2 mg/ug respectively, P <0.002) (Figure 6.14.2). The RNA/protein ratio, an index of the protein synthetic capacity of the tissue, was higher in the alcoholic liver disease patients than in the control subjects (160.29 ± 14.01 ug/mg vs. 137.1 ± 5.7 ug/mg respectively, $P < 0.003$) (Figure 6.14.3).

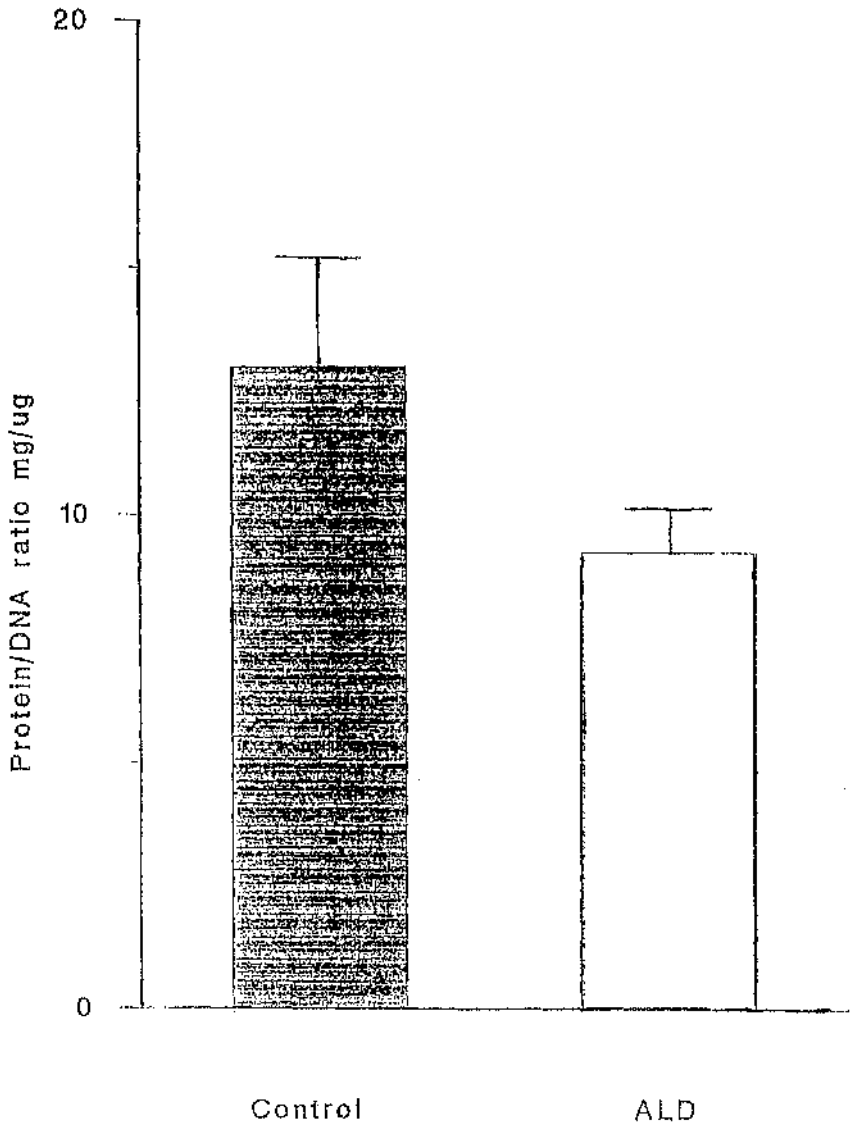
Values of whole-body protein breakdown in alcoholic liver disease and control subjects were similar (204 ± 18 and 196 ± 44 umol leucine/kg/h respectively, P=NS).

Mean leucine (BCAA) concentration was significantly low in alcoholic liver disease (alcoholic liver disease vs. control; 95.99 ± 15.72 umol/l vs. 132.31 umol/l, $P < 0.001$) and mean phenylalanine (aromatic amino acid) concentration



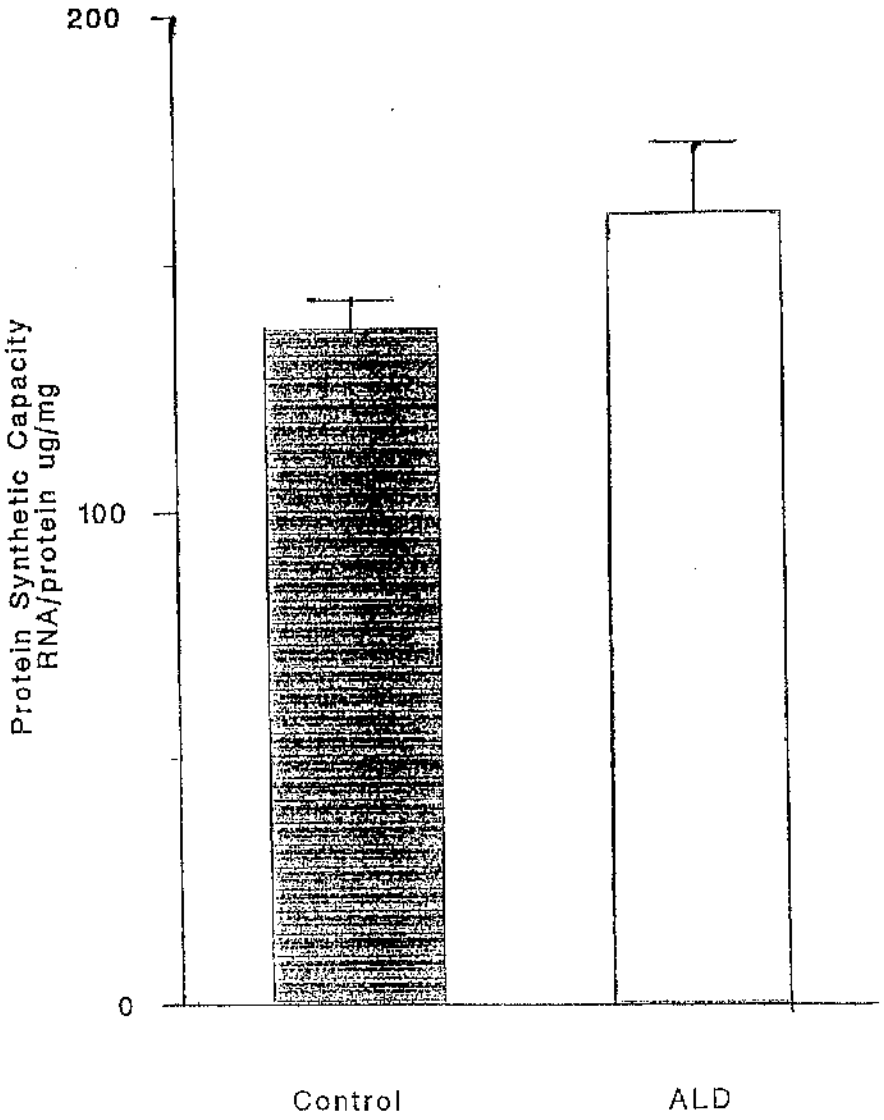
ALD vs. controls, $P < 0.003$

Figure 6.14.1 Rates of small intestinal mucosal protein synthesis in patients with alcoholic liver disease and controls.



ALD vs. controls, $P < 0.002$

Figure 6.14.2 Protein/DNA ratios in patients with alcoholic liver disease and controls.



ALD vs. controls, $P < 0.003$

Figure 6.14.3 Protein synthetic capacities in patients with alcoholic liver disease and controls.

was significantly high in alcoholic liver disease (alcoholic liver disease vs. control; 71.02 ± 6.82 vs. 55.62 ± 4.82 umol/l, $P < 0.001$) (Figure 6.14.4).

The severity of alcoholic liver disease in the patients studied was moderate, average (range), 8.4 (5-13) of a scale 0-18 (Figure 6.14.5).

The severity of alcoholic liver disease correlated positively well with the whole-body protein turnover ($y=119.2+9.7x$, $r=0.88$, $P < 0.01$) and negatively with the rates of small intestinal mucosal protein synthesis rate ($y=2.67-0.07x$, $r=0.72$, $P < 0.05$) (Figure 6.14.6). No correlation was found with the reported amount of alcohol consumed over the years by the patients studied (Figure 6.14.7).

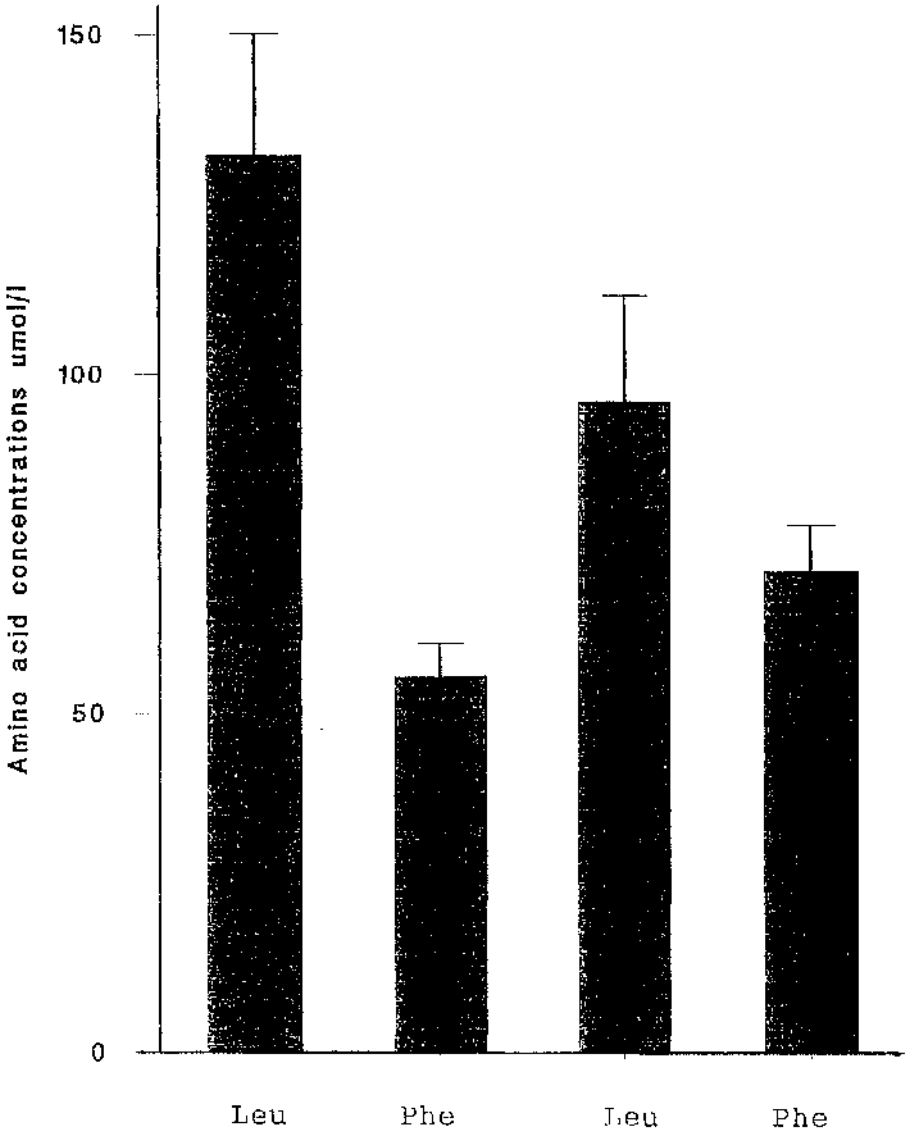


Figure 6.14.4 Blood concentrations of leucine and phenylalanine in patients with alcoholic liver disease and controls.

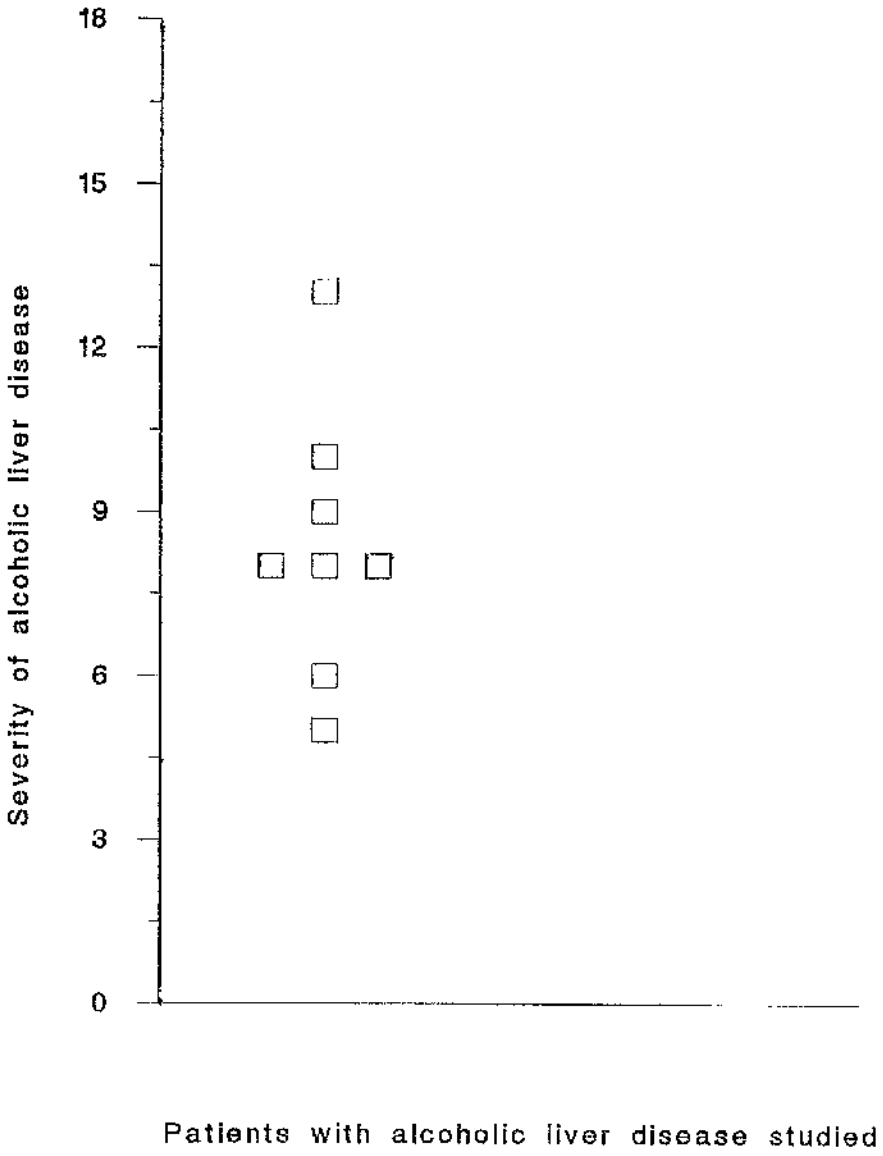
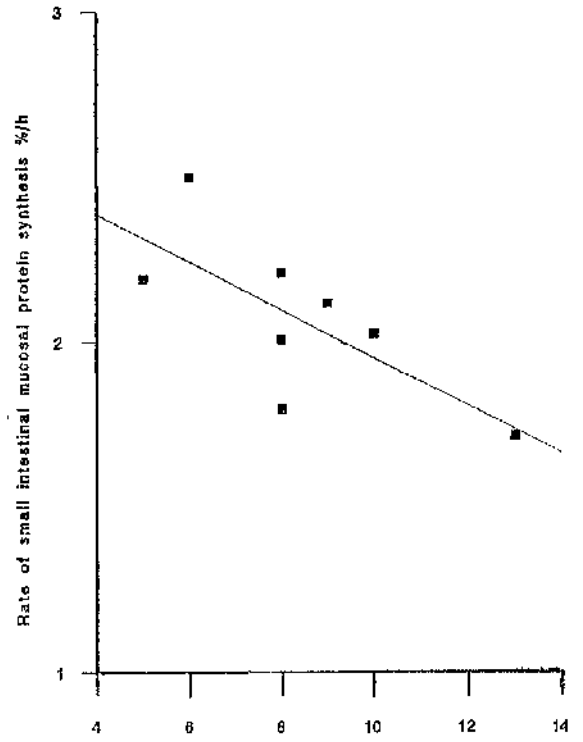
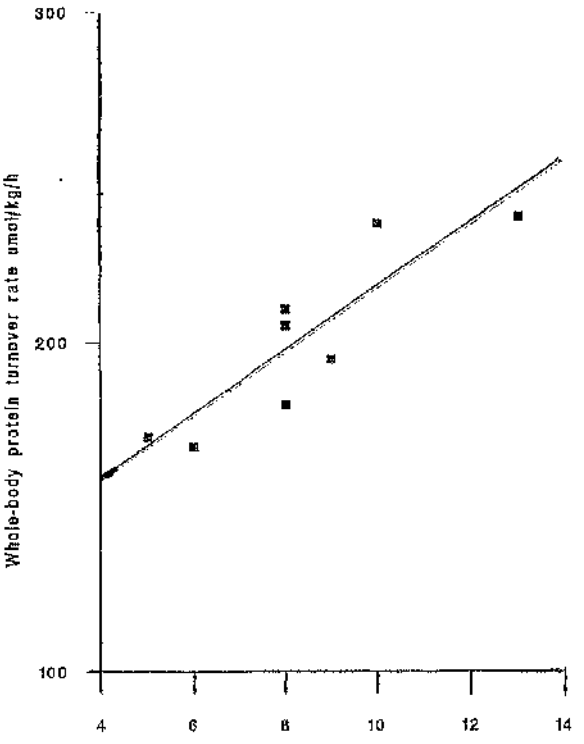


Figure 6.14.5 The severity of alcoholic liver disease in the patients studied.



y=2.67-0.07x,

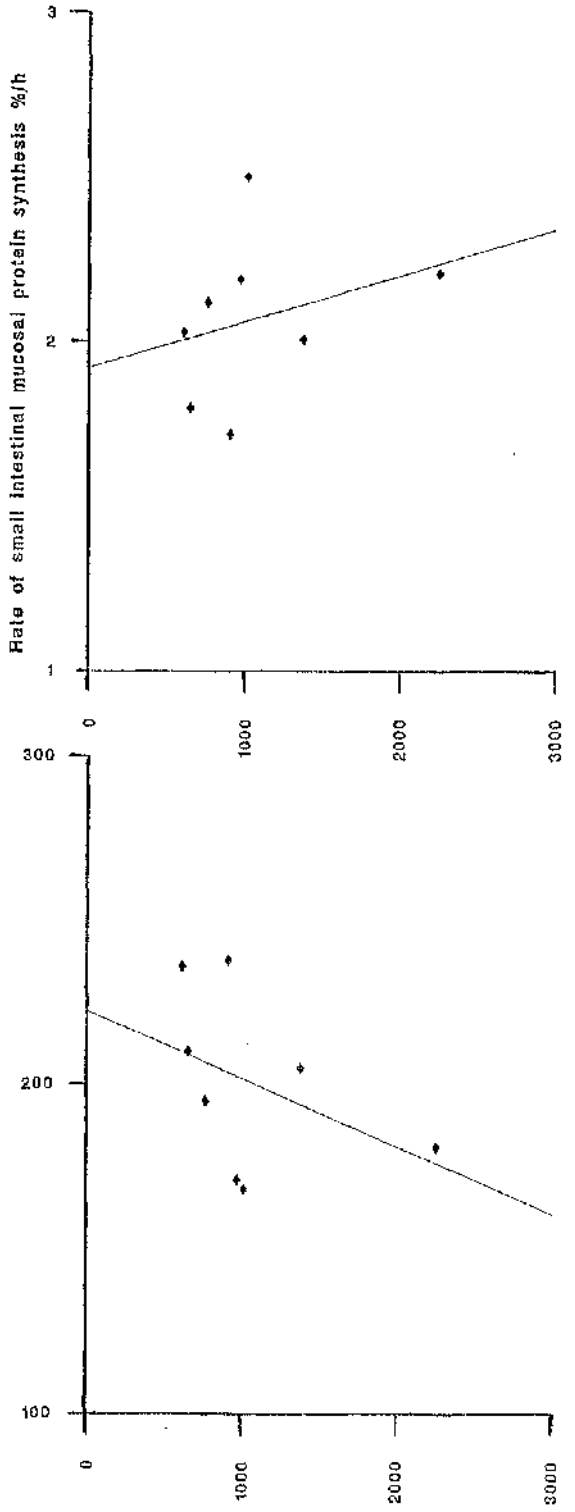
r=0.72, $P<0.05$



y=119.2+9.7x,

r=0.88, $P<0.01$

Figure 6.14.6 The correlation between the severity of alcoholic liver disease and the rates of small intestinal mucosal protein synthesis and whole-body protein turnover.



y=1.92+0.00x,

r=0.31, *P*=NS

y=222.2−0.02x,

r=0.41, *P*=NS

Figure 6.14.7 The correlation between the amount of alcohol consumed and the rates of small intestinal mucosal protein synthesis and whole-body protein turnover.

6.14.5 DISCUSSION

The results of this study showed that small intestinal mucosal protein synthesis was depressed by 21% and this depression is accompanied by a 16% increase in the protein synthetic capacity of the intestinal mucosa and an overall stability of the whole-body protein turnover.

Previous work in liver cirrhosis showed a fall in protein synthesis of other tissues, e.g skeletal muscle, accompanied by an overall fall in whole-body protein turnover (246). In another report the whole-body protein turnover was found to be higher than normal control (247). The change in whole-body protein turnover probably depends on the severity of the liver disease (248). Although in this study there has been no real difference in the whole-body protein turnover between alcoholic liver disease and the controls, but there was a significant, positive correlation between the indices of disease severity and the whole-body protein turnover rate. The rise in the severity of alcoholic liver disease also closely correlated with the reduction in the rate of small intestinal protein synthesis (Figure 6.14.6).

The reduced protein/DNA ratio in alcoholic liver disease suggests a smaller size of the mucosal cells or simply a reduced protein content of the intestinal mucosal tissue as part of the general reduction in the total lean body mass. Patients with significant alcoholic liver disease tend to have a lower protein intake than usual (249) and this poor dietary intake is probably responsible for the alteration in

lean body mass and fat stores found in alcoholic liver disease patients (245).

The protein synthetic capacity is elevated in alcoholic liver disease which could be a response to the catabolic effect of alcohol or to meet the elevated protein requirements in liver disease (247).

Alcohol can induce defects in the intestinal transport of various amino acids in rat, such as phenylalanine, leucine, glycine, alanine, methionine and valine (250,251). These defects might affect the concentration of various amino acids beside the effect of the altered liver metabolism and handling of these amino acids in particular the aromatic amino acids. Plasma clearance does not seem to be a factor in the changes seen in the concentrations of some amino acids such as leucine in alcoholic liver disease (252). The amino acids concentrations in our patients are in keeping with previous reports (252,253,254).

Previous work in our department suggested that in alcoholic patients there is functional impairment of water and electrolytes absorption without associated changes in the small intestinal mucosal histology (255). Animal work suggested a direct damaging effect of alcohol on the intestinal mucosa causing atrophy or shortening of the intestinal villi (256). In our study no abnormality was detected in the small intestinal mucosal histology. Furthermore, we have found that mucosal villous atrophy is associated with elevated rather than reduced rates of mucosal protein synthesis (Chapter 12).

In conclusion, it remains to be established whether the reduced rate of small intestinal mucosal protein synthesis found in alcoholic liver disease is related to poor dietary intake, direct alcohol effect or due to the alcoholic liver disease. Further studies are required to measure small intestinal mucosal protein turnover in alcoholic liver disease after abstinence.

6.14.6 SUMMARY

We have applied a method using stable isotope labelled amino acids to measure small intestinal mucosal protein synthesis to investigate the effect of alcoholic liver disease on the function of the small intestine; we have also measured whole-body protein turnover. Sixteen subjects were studied, 8 normal volunteers (means ± SD) (44 ± 15 y, 71 ± 12 kg, 171 ± 11 cm) and 8 patients with alcoholic liver disease (43 ± 7 y, 74 ± 14 kg, 171 ± 8 cm). All subjects received primed, continuous IV infusions of L-[1-^{13}C]leucine, 1 mg/kg body weight/h after an overnight fast. After 4 h of tracer infusion, distal duodenal biopsies were obtained via endoscopy. Protein synthesis was calculated from protein labelling relative to intracellular leucine enrichment, after appropriate mass spectrometric measurements. Whole-body turnover was calculated from plasma ^{13}C KIC enrichments. Rates of duodenal mucosal protein synthesis were 2.58 ± 0.32 (%h^{-1}, mean ± SD) in the normal subjects vs. 2.04 ± 0.18 in the alcoholic liver disease patients, $P < 0.003$ despite the fact that protein synthetic capacity (ug RNA/mg protein) was

higher in alcoholic liver disease (alcoholic liver disease vs. control; 160.29 ± 14.01 vs. 137.1 ± 5.7 ug/mg, $P < 0.003$); the mucosal cell size, as indicated by the protein/DNA ratio, was lower in alcoholic liver disease (alcoholic liver disease vs. control; 9.23 ± 0.91 vs. 13 ± 2.2 mg/ug; $P < 0.002$). Although the mean rates of whole-body protein turnover were not different between the two groups (alcoholic liver disease vs. control; 201 ± 27 vs. 196 ± 44 umol leucine/kg/h; P=NS) in the alcoholic liver disease patients there was an inverse relationship between rate of intestinal mucosal protein synthesis and extent of severity of alcoholic liver disease ($r = 0.72$, $P < 0.05$) and a direct relationship between rate of whole-body protein turnover and severity of alcoholic liver disease ($r = 0.88$, $P < 0.01$) and a negative correlation with small intestinal mucosal protein synthesis ($r = 0.72$, $P < 0.01$). Thus there was an inverse relationship between rate of small intestinal mucosal protein synthesis and rate of whole-body protein turnover in alcoholic liver disease patients which was not seen in the normal control subjects. The rate of intestinal mucosal protein synthesis was depressed by 21% in alcoholic liver disease despite a 16% increase in the protein synthetic capacity. The lean tissue wasting observed in alcoholic liver disease appears to extend to the gastrointestinal tract, which shows a depressed anabolic activity (despite a sufficient synthetic capacity) in proportion to whole-body breakdown. The functional significance of these changes remains to be established.

SECTION VII

APPLICATION OF THE TECHNIQUE OF SMALL INTESTINAL MUCOSAL PROTEIN SYNTHESIS IN PATIENTS WITH ILEOSTOMY

CHAPTER 15

REGIONAL VARIATION IN THE RATES OF SMALL INTESTINAL MUCOSAL PROTEIN SYNTHESIS IN SUBJECTS WITH ILEOSTOMY

7.15.1 INTRODUCTION

The small intestine extends from the pylorus to the ileocaecal valve and measures about 5 m (3.5-6.5 m) in length. It is divided into 3 regions; the duodenum is the first 25 cm, the jejunum is the proximal 40% and the ileum is the distal 60% of the remaining small bowel. There is no recognized line of division between the jejunum and the ileum. There are anatomical, structural, histological and functional differences between these three regions of the small intestine (206,257).

In the intestinal mucosa, cell function is different at different levels; the physiology of absorption is not the same in the jejunum and ileum (9), the rates of cell renewal are different, and it is likely that rates of protein turnover are also different (37). Recently there has been a considerable interest in human gastrointestinal protein metabolism (39,192) and work in animal preparations suggested that the small intestine contributes significantly to the whole-body protein turnover in spite of it's relatively small contribution to whole-body protein mass (193). No data are available on the comparability of the rates of mucosal protein synthesis between the various regions of the small intestine.

We have developed a safe, reproducible and reliable method to measure protein synthesis in the gastrointestinal mucosa using BCAAs labelled with ^{13}C (Chapter 10) and used it to address this question.

7.15.2 AIMS

The main aims of this study were; 1) to measure the rate of mucosal protein synthesis in the jejunum and ileum of ileostomy subjects, 2) to investigate possible differences between the rates of protein synthesis in these regions of the small intestine, and 3) to assess the effect of ileostomy on the whole-body protein turnover.

7.15.3 PATIENTS AND METHODS

Six subjects with ileostomy, three men and three women age 27-71 years, weight 54-84 kg were studied (Table 7.15.1). Each subject gave written consent after a full explanation of the nature of the study. Approval for the studies was obtained from the local Ethics Committee of Glasgow Royal Infirmary University NHS Trust.

Patients with ileostomy were recruited to allow access to the terminal ileum. Five of them had undergone total colectomy for ulcerative colitis and one patient had colonic inertia and still has her colon in situ though she did have an ileostomy.

TABLE 7.15.1 Characteristics of patients with ileostomy studied for small intestinal mucosal protein synthesis.

Patient	Age-Y	Sex	Weight-kg	Height-cm	%Ideal BW
1	68	F	65	159	113.04
2	72	M	84	170	125.19
3	35	M	51	165	78.95
4	70	M	83	172	121.88
5	31	F	54	172	83.72
6	27	F	64	177	95.24

% ideal BW : % ideal body weight (198)

Results obtained from a group of 8 normal volunteer subjects (mean ± SD) (44 ± 15 y, 71 ± 12 kg, 171 ± 11 cm) were used as controls (Table 6.14.2).

L-[1-^{13}C]leucine 99 Atom % was dissolved in sterile, non-pyrogenic 0.9% NaCl solution (150 mmol/liter) immediately before administration and was sterilized by passage through a 0.20 micron filter.

The subjects were studied in the morning after an overnight fast. Venous blood samples were taken, centrifuged and separated, plasma was kept in liquid nitrogen. A priming dose of L-[1-^{13}C]leucine 1mg/kg body weight was given IV over one min. A constant infusion of the same tracer at a rate of 1mg /kg body weight/h using 120 ml of 0.9% NaCl was then continued for 4 h. The same rate of

infusion was used in both groups and was kept constant during the time of infusion. After 4 h of continuous infusion small intestinal mucosal samples were obtained from the jejunum and the ileum. The jejunal biopsies were obtained using a Crosby Kugler capsule (113) which was introduced 2 h after the start of the tracer infusion. The position of the capsule was about 10 cm distal to the ligament of Trietz and was verified radiologically. The range of pooled wet weight of jejunal biopsies was 11.1-22.1 mg. Immediately after taking the jejunal biopsy, ileoscopy was performed and multiple ileal biopsies obtained for ^{13}C analysis and histology. The range of pooled wet weight of ileal biopsies was 60.0-79.8 mg. Venous blood samples were taken at -15, 0, 60, 120, 180 and 240 min respectively and separated plasma kept in liquid nitrogen with the biopsies.

The labelling of plasma leucine and KIC was measured by means of standard GCMS techniques using t-BDMS derivatives **(199)**. Mucosal tissue samples were frozen in liquid nitrogen immediately on sampling, and samples pooled for storage at -70^0C. Plasma protein from the pre-infusion samples was used to estimate basal body protein ^{13}C labelling for use in the calculation of mucosal protein synthetic rate. The labelling with ^{13}C of leucine in hydrolysed protein using preparative gas chromatography and isotope ratio mass spectrometry of the CO_2 liberated by ninhydrin (185).

The rates of small intestinal mucosal protein synthesis and whole-body protein breakdown were calculated as

previously described (Chapter 9). Values were expressed as means ± SD. Statistical analysis used Student t-test for the non parametric, unpaired data. Differences were considered significant at P values of < 0.05.

7.15.4 RESULTS

The plasma leucine enrichment reached a plateau of 7.12 ± 0.57 APE in the ileostomates and 5.11 ± 0.56 APE in the control subjects; ileostomates vs. control, $P < 0.005$, within one h of IV L-[1-^{13}C]leucine infusion and remained so throughout the infusion period until biopsies were obtained (Figure 7.15.1). The KIC enrichment was also higher in the ileostomy patients than in the controls, ileostomates vs. controls; 5.32 ± 0.59 vs. 3.82 ± 0.53, $P < 0.005$ (Figure 7.15.2). There was no difference in the KIC/leucine ratio between the two groups, controls vs. ileostomates; 0.76 ± 0.07 vs. 0.75 ± 0.04, P = NS.

The rates of jejunal and ileal mucosal protein synthesis were 2.14 ± 0.20 and 1.20 ± 0.20 $\%h^{-1}$ respectively, $P < 0.001$ (Figure 7.15.3).

The rate of whole-body protein turnover was lower in the ileostomates, ileostomates vs. controls; 145 ± 15 vs. 196 ± 44 umol/kg/h, $P < 0.01$ (Figure 7.15.4).

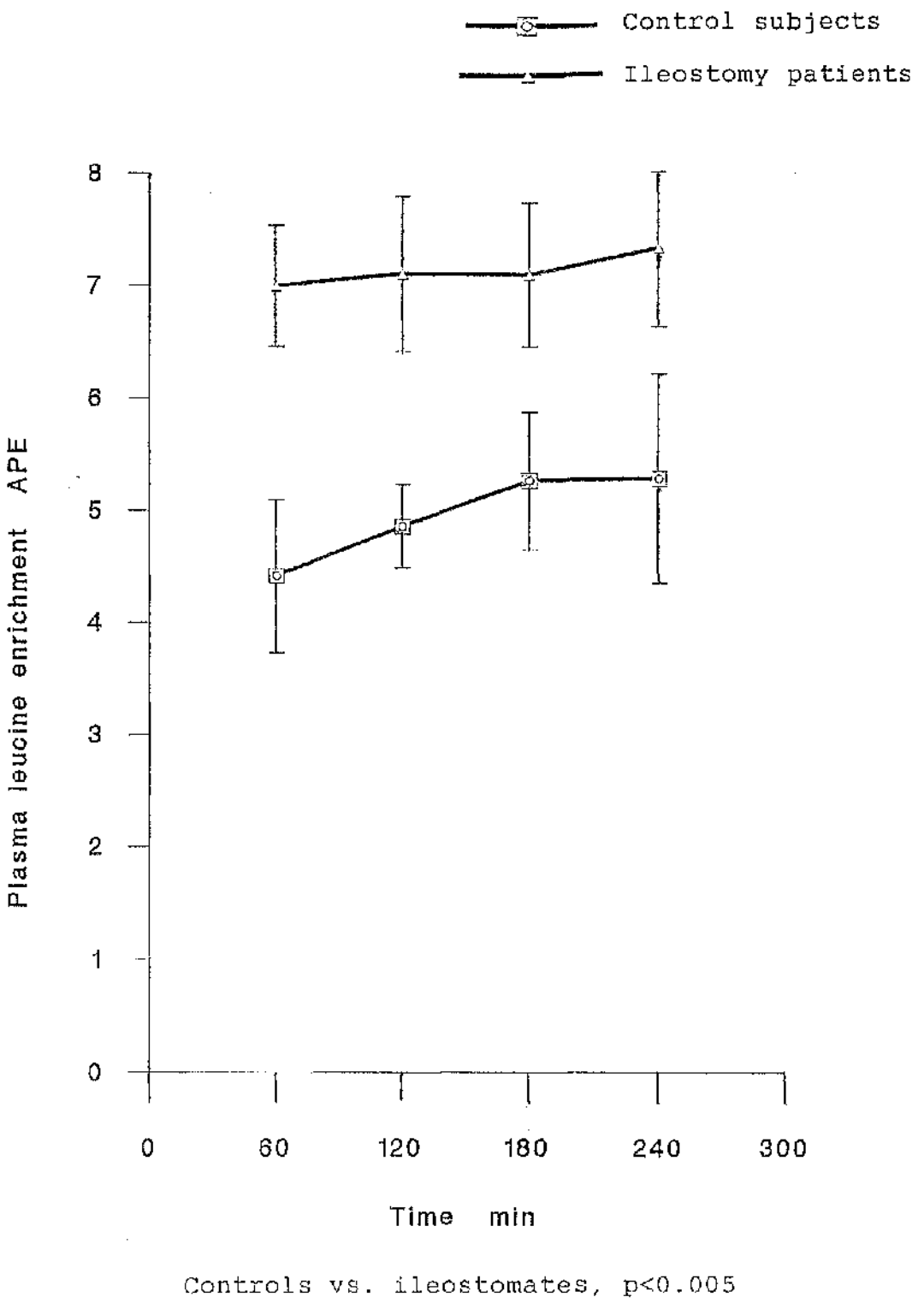
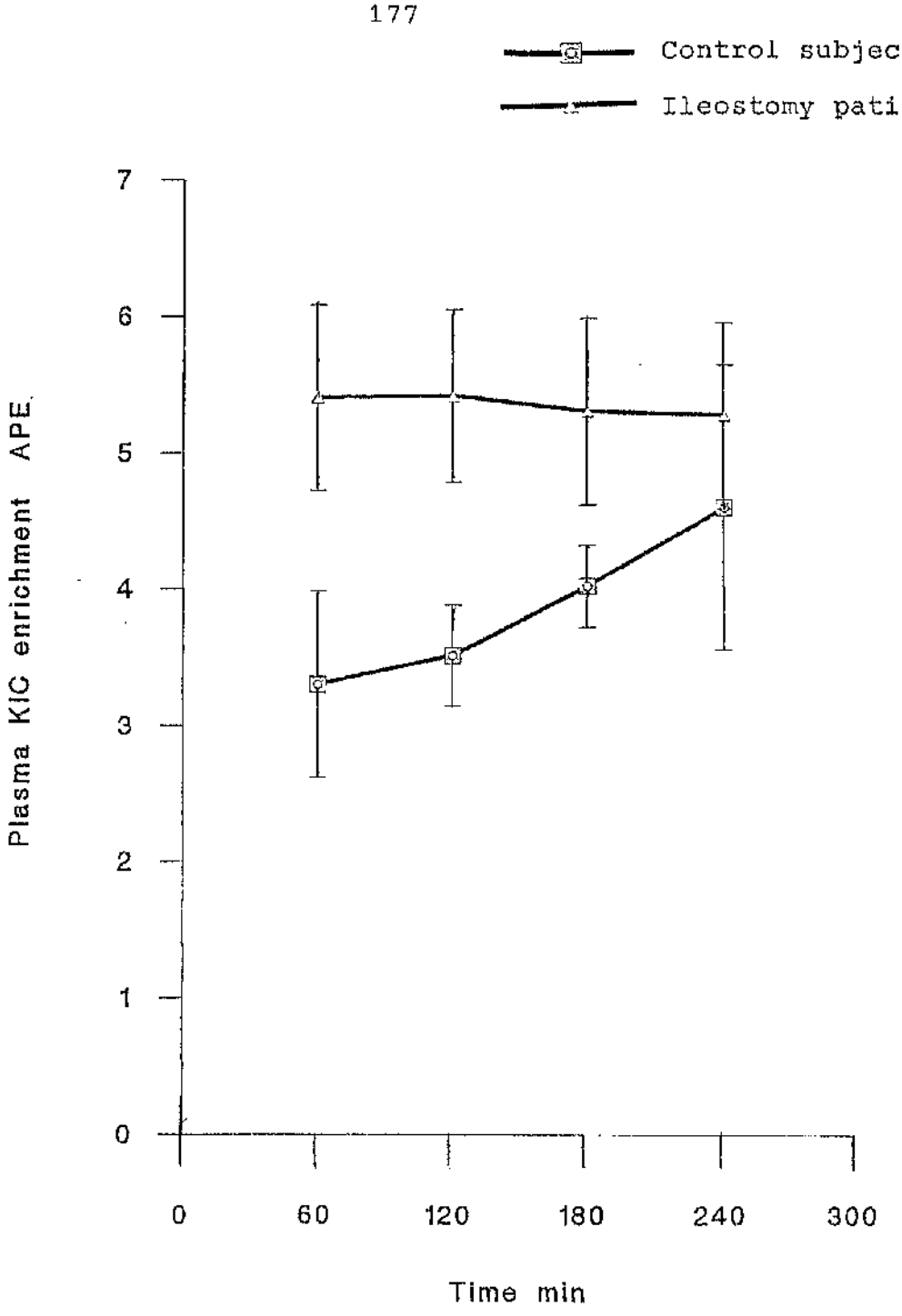
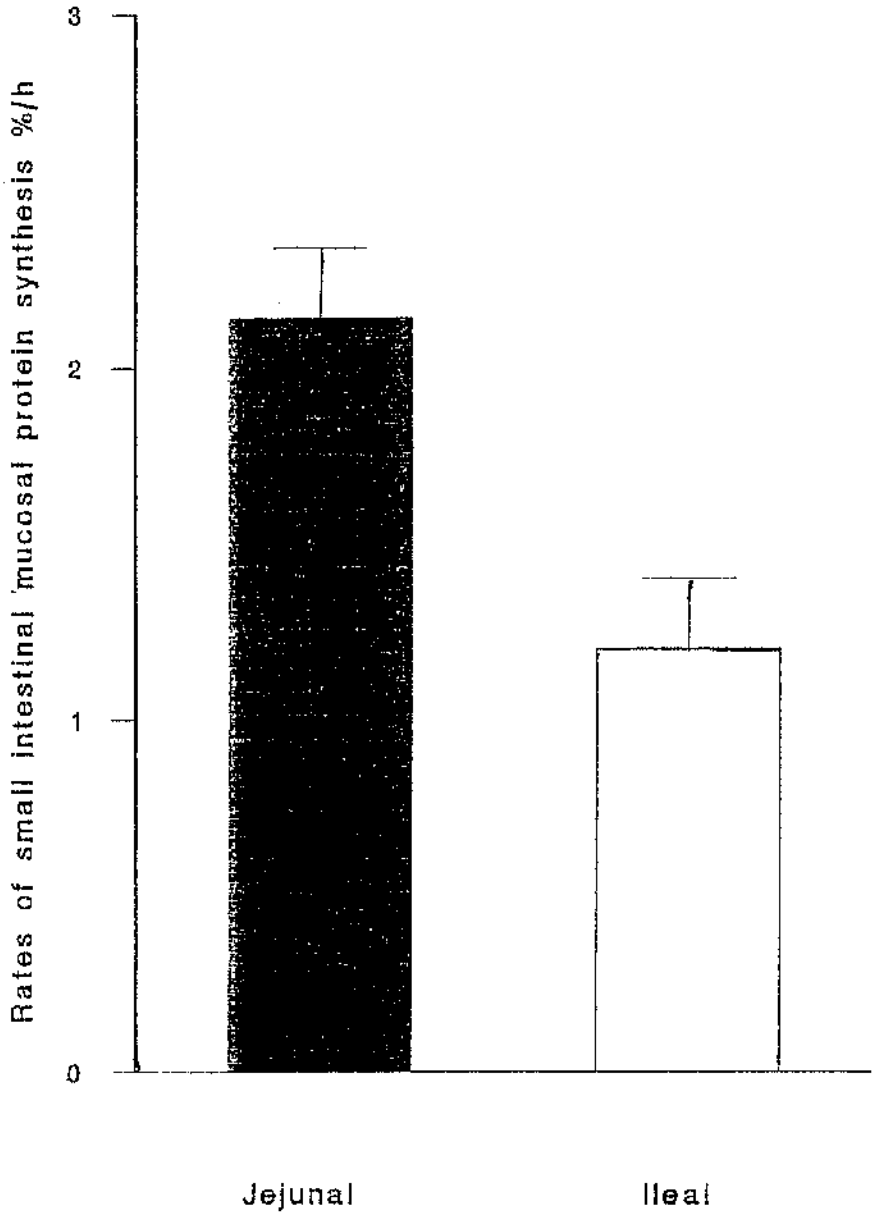


Figure 7.15.1 Plasma leucine enrichment in the control subjects and ileostomy patients.



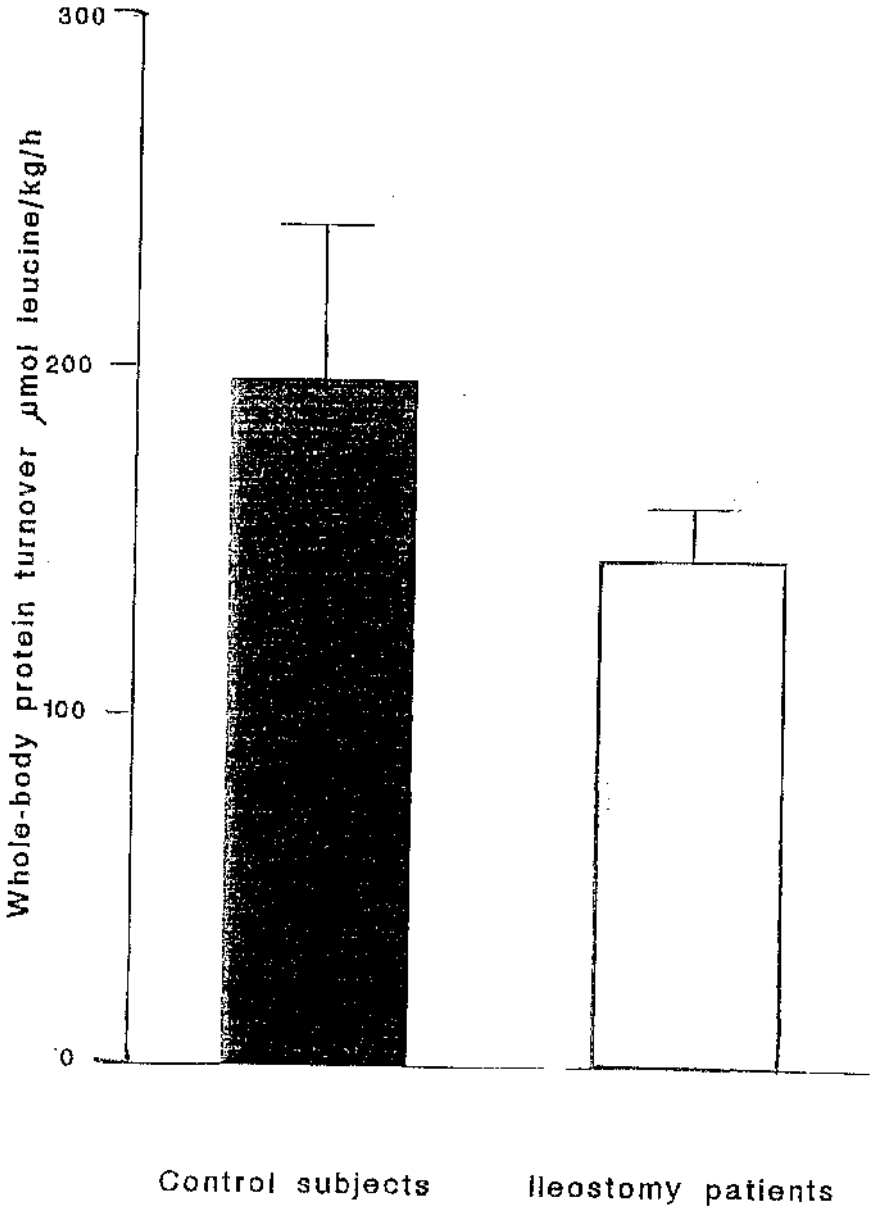
Controls vs. ileostomates, $p<0.005$

Figure 7.15.2 Plasma alpha-ketoisocaproate enrichment in the control subjects and ileostomy patients.



Jejunal vs. ileal, $P < 0.001$

Figure 7.15.3 Jejunal and ileal mucosal synthetic rates in the ileostomy subjects



Controls vs. ileostomates, $P < 0.01$

Figure 7.15.4 Rates of whole-body protein turnover in normal control subjects and in patients with ileostomy.

7.15.5 DISCUSSION

The results of this study show that the fractional rate of protein synthesis in the various regions of the small intestine is not the same in ileostomates. The rate of protein synthesis in the jejunal mucosa is substantially higher than the rate in the ileal mucosa. The results strongly suggest that the rate of protein synthesis in the ileal mucosa is half that in the duodenal mucosa of normal subjects (Chapter 10), and about 25 times greater than the rate of protein synthesis observed in skeletal muscles (175,204).

The ileal mucosal biopsies from the patient who had ileostomy showed normal mucosal pattern on histological examination and the pathological reports on the surgical specimens of the resected colons as well as the case records of those patients were reviewed and the diagnosis of ulcerative colitis was verified. The patient with colonic inertia had a normal colonoscopy and upper gastrointestinal endoscopy with normal histology of the mucosal biopsies. In this subject the ileoscopic biopsies were taken from various areas and sent for histology and from about 10 cm and 60 cm away from the stoma for protein synthesis measurement. The rate of protein synthesis in the distal ileal mucosa was 1.05 $\%h^{-1}$ and in the proximal ileal mucosa 1.22 $\%h^{-1}$ which further confirmed the fact that the distal part of the small

intestinal mucosa has a lower rate of protein synthesis than the proximal part.

All subject studied were within normal nutritional status and had not made any major modifications to their diet as a result of the ileostomies. On one occasion a subject did not tolerate the Crosby Kugler capsule and on another occasion the capsule failed to fire, hence we only had 4 jejunal mucosal samples and 6 ileal mucosal samples.

Ileostomy appears to be associated with a reduction in the whole-body protein turnover and increased values for the tracer amino acid and ketoacids plasma enrichments despite using the same rate of tracer infusion which was kept constant in both groups. The explanation of this is not fully understood, whether it is related to reduced lean tissue mass following colectomy, leucine compartmental changes or changes in the efflux of leucine and KIC from various organs in the body, remains to be established. However, this observation does indicate that the colon contributes significantly to the whole-body protein turnover.

There were attempts to avoid permanent ileostomy and establish a normal route of evacuation by ileo-rectal anastomosis (258) and more recently, over the past 15 years or so, by the reservoir operation to create a pelvic pouch and maintain the voluntary control over evacuation (259,260). There has been reports on the morphological, histochemical and functional changes in the ileal mucosa

following such operations (261,262,263). Pouchitis; varying degrees of villous atrophy with inflammation has become the most important long term complication affecting pelvic ileal pouches (262). The physiological mechanisms underlying pouch adaptation and inflammation are poorly understood. The method we used to measure the rate of protein synthesis in the ileal mucosa has a potential role in the investigation of these patho-physiological changes.

Further studies are required to measure the duodenal mucosal protein synthesis in ileostomates and compare this with the results of the duodenal mucosa obtained from normal volunteers.

7.15.6 SUMMARY

We have applied a robust and reproducible method to measure protein synthesis in the gastrointestinal mucosa to investigate possible differences in the rates of mucosal protein synthesis between the proximal and distal regions of the small intestine in ileostomates and to assess the effect of ileostomy on protein turnover. Six subjects with ileostomy were studied (mean ± SD) (46 ± 21 y, 65 ± 14 kg, 169 ± 6 cm). Results from a group of 8 normal volunteer subjects (44 ± 15 y, 71 ± 12 kg, 171 ± 11 cm) were used for comparison. The presence of ileostomy in these subjects allowed practical access to the distal region of the small intestine. The terminal ileum was histologically normal in all ileostomy subjects studied. All subjects received

primed, continuous IV infusions of L-[1-^{13}C]leucine after an overnight fast. After 4 h of tracer infusion jejunal biopsies were obtained using Crosby Kugler capsule and ileal biopsies were obtained via endoscopy at the same time. Protein synthesis was calculated from protein labelling relative to intracellular leucine enrichment, after appropriate MS measurements. The results are presented as means ± SD. Rates of jejunal and ileal mucosal protein synthesis were 2.14 ± 0.2 and 1.2 ± 0.2 (%h^{-1}) respectively; jejunal vs. ileal $P < 0.001$. Plasma leucine enrichment was, ileostomates vs. controls; 7.12 ± 0.57 vs 5.11 ± 0.56 APE, $P < 0.005$ and plasma KIC enrichment was, ileostomates vs. controls; 5.32 ± 0.59 vs. 3.82 ± 0.53 APE, $P < 0.005$. The whole-body protein turnover rate was, ileostomates vs. controls; 145 ± 15 vs. 196 ± 44 umol/kg/h, $P < 0.01$. In the small intestinal mucosa of subjects with ileostomy the rates of protein synthesis were different at different levels, being significantly higher at the proximal level and lower at the distal level. The plasma leucine enrichment increased by 28% and plasma KIC enrichment increased by 26% with a significantly reduced whole-body protein turnover. These changes are probably related to the reduction in the size of the plasma amino acid compartment or reduced lean tissue mass following colectomy and indicate that the colon has a significant contribution to the whole-body protein turnover.

S E C T I O N V I I I

D I S C U S S I O N

DISCUSSION

The fractional rates of protein synthesis observed in the normal duodenal mucosa were high at 2.5 $\%h^{-1}$, being some 25-50 times greater than those previously observed for skeletal muscle (175,204) and also about two to three times greater than those determined for normal colonic mucosa (39,173,194). Despite the substantial differences in the extent of labelling of the intracellular and plasma pool of leucine and valine when the tracers were administered IG or IV, and despite the substantial differences in the absolute extent of labelling of mucosal protein, the rate of mucosal protein synthesis calculated on the basis of the intracellular amino acid pool labelling were identical.

The results suggest that in the post-absorptive state, there is no detectable preferential channelling of amino acids into protein synthesis from the apical or basolateral membrane of the small intestinal mucosal cells. The lower plateau values for plasma labelling for the tracers infused via the IG route than the IV route suggested that the IG tracers were being diluted to a greater extent by amino acids released from protein breakdown, most likely in the splanchnic tissue. The results suggest that there is little transamination of the BCAAs during their passage from the gastrointestinal lumen into the peripheral blood in accordance with the known low capacities of the gut and liver for BCAA transamination (203).

It is impossible to exactly quantify the total amount of small intestinal mucosal protein in order to calculate the absolute amount of protein synthesis contributed by the gastrointestinal tract but the high rate suggests that mucosal protein synthesis is a major contributor to whole body protein turnover.

Leucine tracer was incorporated into gastrointestinal protein to an extent proportional to the time of exposure of the tissue to the tracer at least when delivered IV. We have assumed that this is also the case for the tracers delivered IG. Therefore the k_s calculated in this infusion protocol is equal to the FSR when using the expression K_s $(\%h^{-1})$ = $(E_t - E_0/E_p)$ X $1/t$ X 100.

The effect of untreated coeliac disease on the rate of small intestinal mucosal protein synthesis *in vivo* was determined in this work. The rate of protein synthesis by the small intestinal mucosal cells was higher in coeliac patients in spite of the smaller size of the enterocytes. The increased rate of mucosal protein synthesis in coeliac patients is in agreement with the results of an *in vitro* study by Jones et al.(209). Presentation of the tracer amino acid to the apical (luminal) side of the enterocytes seems to be associated with higher protein synthetic rate than on presentation of these tracers on the basolateral (intravascular) side. This finding was unexpected and is not seen in normal healthy subjects, probably due to the preferential channelling of amino acids from the luminal side

because of the immaturity of the enterocytes in coeliac disease. In coeliac disease there is evidence of increased mucosal cell turnover with marked proliferation of normal crypt cells (212), and our findings are in agreement with this. The KIC/Leu and KIV/Val ratios were significantly low in coeliac patients suggesting more dilution of the keto acids resulting from elevated rate of protein breakdown and turnover. The intestinal mucosa showed evidence of atrophy as manifested by the decreased protein/DNA ratio. The capacity of protein synthesis (RNA/protein) is elevated in the coeliac patients. This is probably a reflection of the immaturity of the enterocytes lining the villus and/or elevated rate of mucosal protein breakdown. The contribution of the lamina propria to the protein synthesis of the mucosa is negligible and most of the protein synthesis takes place in the enterocytes and only negligible amounts synthesised by the cells of the lamina propria (141).

Intestinal biopsy is of central importance in the investigation of coeliac disease. Information on the histomorphometric characteristics of the crypts cells in coeliac disease is lacking apart from the mention of high mitotic index (212,231). This study showed significant increase in the nuclear surface area in the coeliac disease patients which is probably a reflection of increased mitotic activity. The change in the nuclear surface area of the crypt cell following the introduction of gluten free diet provides an early indicator of the response to this therapy in coeliac disease. We found a significant reduction in the

surface area of the crypt cell's nuclei in patients who showed clinical as well as histological response to gluten free diet. The results of the histomorphometric study suggests that the nuclear surface area of the crypt cell in coeliac disease is affected by the folate status and not the ferrous status.

The results of the study in alcoholic liver disease showed that the rate of small intestinal mucosal protein synthesis was depressed by 21%, despite a 16% increase in protein synthetic capacity. There was an overall stability of the whole-body protein turnover. The change in whole-body protein turnover probably depends on the severity of the liver disease (248). I have found a significant, positive correlation between the indices of disease severity and the whole-body protein turnover rate. The rise in the severity of alcoholic liver disease also closely correlated with the reduction in the rate of small intestinal protein synthesis. The reduced protein/DNA ratio in alcoholic liver disease suggests a smaller size of the mucosal cells or simply a reduced protein content of the intestinal mucosal tissue as part of the general reduction in the total lean body mass. The protein synthetic capacity is elevated in alcoholic liver disease which could be a response to the catabolic effect of alcohol or to meet the elevated protein requirements in liver disease (247). Animal work suggested a direct damaging effect of alcohol on the intestinal mucosa causing atrophy or shortening of the intestinal villi (256). In our study no

abnormality was detected in small bowel mucosal histology. It remains to be established whether the reduced rate of small intestinal mucosal protein synthesis found in alcoholic liver disease is related to poor dietary intake, direct alcohol effect or due to the alcoholic liver disease.

The fractional rate of protein synthesis in the various regions of the small intestine is not the same in ileostomates. The rate of protein synthesis in the jejunal mucosa is substantially higher than the rate in the ileal mucosa. The results strongly suggest that the rate of protein synthesis in the ileal mucosa is half that in the duodenal mucosa of normal subjects, and about 25 times greater than the rate of protein synthesis observed in skeletal muscles (175,204). Ileostomy appears to be associated with a reduction in the whole-body protein turnover and increased values for the tracer amino acid and ketoacids plasma enrichments despite using the same rate of tracer infusion which was kept constant in both groups. The explanation of this is not fully understood, whether it is related to reduced lean tissue mass following colectomy, leucine compartmental changes or changes in the efflux of leucine and KIC from various organs in the body, remains to be established.

SECTION IX

CONSIDERATIONS ON FUTURE APPLICATIONS OF STABLE ISOTOPES AND MUCOSAL PROTEIN SYNTHESIS TECHNIQUE

CONSIDERATIONS ON FUTURE APPLICATIONS OF STABLE ISOTOPES AND MUCOSAL PROTEIN SYNTHESIS TECHNIQUE

The present method for the measurement of protein synthesis has applications in various tissues and organs in the body, as long as these tissues and organs are capable of being biopsied or approached peroperatively.

Of particular interest is to study the physiological response of the gastrointestinal mucosa to nutrient supply either enterally or parenterally. The response of whole-body protein dynamics to food depends on the previous dietary history especially the amount of protein in the diet (264). In skeletal muscle, the response to food is similar in direction, but probably greater in extent, than that in the whole-body; that is, protein synthesis increases and protein breakdown falls during feeding (265,266,267). The effect of feeding on intestinal protein synthesis is not known and the reports available on this subjects are inconclusive (268,269). Therefore this method could be used to measure the effect of feeding, both enteral and parenteral, on small intestinal protein synthesis. This is an important aspect which has implications for the preservation of the function of the gastrointestinal tract during enteral and parenteral feeding. The extent to which human mucosal protein synthesis can be modulated by physiological influences including nervous, hormonal and nutritional effects, remains to be determined.

Further developments of the current technique of measuring mucosal protein synthesis *in vivo* can be expected to allow

the contribution of each cell type to the rate of protein synthesis in the whole biopsy to be determined.

Another application is to reevaluate the rate of protein synthesis in patients with coeliac disease who have responded clinically and histologically to a gluten free diet as well as in those patients who have not responded to this diet. It has been found that the rates of *in vitro* protein synthesis by treated non-responsive coeliac mucosa significantly lower than the rates in the untreated coeliac mucosa but greater than the rates in the control mucosa (209).

Further application of the method described here is to study the small intestinal protein turnover in alcoholic liver disease after abstinence to see if this has any effect on the intestinal protein metabolism.

Pouchitis is the most important long term complication affecting pelvic ileal pouches (262), and the physiological mechanisms underlying pouch adaptation and inflammation are poorly understood. The method we used to measure the rate of protein synthesis in the ileal mucosa has a potential role in the investigation of these patho-physiological changes.

To evaluate the effect of ileostomy on the rate of small intestinal protein synthesis further study is required to measure the duodenal mucosal protein synthesis and to compare it with the results of the duodenal mucosa obtained from normal volunteers.

Various other gastrointestinal diseases can be studied on the same line such as Crohn's disease using the method described in this thesis to evaluate the rates of mucosal

protein synthesis before and after certain treatment modalities such as steroids and elemental diet.

The latest development of very accurate mass spectrometers in recent years have extended the range of work which is possible with stable isotopes. Because of this accuracy and the safety of using stable isotopes comparing with radioactive isotopes, we have developed a new method for the measurement of gastrointestinal mucosal protein synthesis which can be added to the armament of research and investigative gastroenterology. Various applications of stable isotopes have already been mentioned (Chapter 4), in addition some other applications merit a particular attention.

The use of tracer amino acids to measure the absorptive capacity of the small intestine and the rate of absorption in normal subjects and in patients with malabsorption such as short bowel syndrome. Such test would be minimally invasive technique and it has it's applications in clinical practice to evaluate protein absorption in various disease entities. The technique of stable isotope labelling also has a potential wide range of applications in the study of absorption of various nutrients, trace elements and drugs and to evaluate the influence of various factors on their absorption. In generalized malabsorption there is almost always impaired fat absorption, and the most reliable test is the quantitative faecal fat analysis (206), such as dual isotope fat absorption (DIFA) test (270,271). This test employs radioactive isotopes which could be replaced by

stable isotopes. The classic test for malabsorption commonly used in clinical practice to assess the absorptive capacity of the small intestine is the D-xylose absorption test (272). This test is flawed with problems and potential uncertainty regarding the accuracy of its results (11,273,274), therefore there is a need for more sensitive and specific absorptive tests.

Another application is to use ^{13}C-acetate breath test to monitor gastric emptying rate in patients with gastrointestinal motility disorders and to follow up the effect of treatment in these patients. There have been attempts to use ^{13}C-glycine and ^{14}C-octanoic acids to measure gastric emptying (100,101,102). This test is safer than the radioscintigraphy, which is the current method used in clinical practice, thereby reducing radiation exposure to the patient. It is also a valid, non-invasive technique. In addition breath tests are easy to perform, even for the elderly or disabled patients and can be carried out at the bedside or even outside the hospital. Breath samples can be analysed after completion of the sampling at a convenient time and can easily be repeated to monitor response to therapy. It is very cost effective method in this time when cost is an important issue.

In conclusion, the development and current applications of stable isotope technology has provided much valuable and practical information which will in time help to develop more effective and practical techniques in clinical investigation and research.

SECTION X

CONCLUSIONS

CONCLUSIONS

The principle aims of the thesis have been achieved.

A new, robust, safe, reproducible and reliable method for the measurement of gastrointestinal mucosal protein synthesis has been developed. This method employs the use of amino acid tracer incorporation principle for the measurement of protein synthesis. The delivery of tracer amino acid was by following the primed-constant infusion rather than the flooding dose technique.

In normal subjects there was no preferential channelling of the tracer amino acid when it was delivered via the IV or the IG route. The incorporation of tracer amino acid in the gut mucosal protein was linear with the exposure time.

In untreated coeliac disease patients the rates of duodenal mucosal protein synthesis were elevated for both IV and IG routes. In this group of patients I found that the IG route was associated with higher rates of gut mucosal protein synthesis than the IV route, for reasons remain to be established. This variation was not found in normal subjects. There was evidence of reduced mucosal cell size and increased protein synthetic capacity in coeliac patients. The mucosal cellular atrophy was confirmed by the histomorphometric study which also showed evidence of increased crypt cell nuclear surface area associated with reduced serum folate levels.

In the alcoholic liver disease patients the rate of small intestinal protein synthesis was depressed despite the increase in the protein synthetic capacity of the intestinal mucosa. The whole-body protein turnover rates were the same in the alcoholic liver disease patients and the control subjects. However, there was a positive correlation with the indices of disease severity which correlated inversely with the rates of mucosal protein synthesis.

In the ileostomy subjects there was a regional variation in the rates of small intestinal mucosal protein synthesis, being higher proximally and lower distally. The rates of whole-body protein turnover were lower in the ileostomates and the enrichment of the tracer amino acid reached higher levels.

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PUBLICATIONS ARISING FROM THE THESIS

1. Nakshabendi IM, Obeidat W, Russell RI, Downie S, Smith K, Rennie MJ. Gut mucosal protein synthesis measured using intravenous and intrgastric delivery of stable tracer amino acids. Am J Physiol 1995;269 (Endocrinol Metab 32): E996-E999.

2. Nakshabendi IM, Downie S, Russell RI, Rennie MJ. Elevated rates of duodenal mucosal protein synthesis *in vivo* in patients with untreated coeliac disease. Gut 1996;38: (accepted).

3. Nakshabendi IM, Shlebak A R, Lee PD, Russell RI. Crypt cell histomorphometry in coeliac disease: comparison with the DNA and protein mucosal composition and correlation with folate and vitamin B12 status. J Clin Pathol 1996 (submitted).

4. Nakshabendi IM, Downie S, Russell RI, Rennie MJ. Small intestinal mucosal protein synthesis is depressed in patients with alcoholic liver disease and correlates with whole-body protein breakdown. Clin Sci 1996 (submitted).

5. Nakshabendi IM, Downie S, Russell RI, Rennie MJ. Regional variation in the rates of small intestinal mucosal protein synthesis in ileostomates. Clin Nutr 1996 (under prparation)

PRESENTATIONS AT LEARNED SOCIETIES' MEETINGS

I have presented the results of various studies during the course of this work, both orally and in the form of posters, at meetings of various learned societies. The abstracts appeared in the proceeding of these meetings.

1. Scottish Society of Experimental Medicine (SSEM)

2. Caledonian Society of Gastroenterology

3. British Society of Gastroenterology (BSG)

4. British Association of Parenteral and Enteral Nutrition (BAPEN)

5. European Society of Parenteral and Enteral Nutrition (ESPEN)

6. Digestive Disease Week, American Gastroenterology Association (DDW, AGA)